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Expression of rag2 and V(D)J Recombinase Activity are Reduced in Aged Mice as a Result of Changes in the Bone Marrow Microenvironment: a Dissertation

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EXPRESSION OF *rag2* AND V(D)J RECOMBINASE ACTIVITY ARE REDUCED IN AGED MICE AS A RESULT OF CHANGES IN THE BONE MARROW MICROENVIRONMENT

A Dissertation Presented

By

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ABSTRACT

Both humans and mice display an age-related decline in immunity. Reduced generation of mature B cells may be a contributing factor due to reduced entry of mature B cells with novel B cell receptors and specificity for pathogens into the mature B cell pool. In aged mice the numbers of B cell precursors within the bone marrow are diminished; there is a severe reduction in numbers of pre-B cells and an increase in numbers of re-circulated mature B cells. Other defects in developing B cells include reduced expression of *rag1* and *rag2* when measured in total bone marrow precursor populations. In the pro-B cell stage of development *rag* expression is essential to the process of V(D)J recombination and the generation of pre-B cells. It was not known prior to this work if *rag* levels were lower in pro-B cells. In Chapter 2 I show that *rag2* expression is reduced in pro-B cells of aged mice. The reduction in *rag2* expression is correlated with a loss of V(D)J recombinase activity in pro-B cells and reduced numbers of pre-B cells. This suggests that in aged mice the reduction in *rag2* expression is sufficient to result in reduced V(D)J recombinase activity and reduced generation of pre-B cells, thus contributing to fewer pre-B cells in aged mice. Furthermore, I have shown that the loss of *rag2* expression and recombinase activity in pro-B cells are the result of age-associated defects in the bone marrow-microenvironment as opposed to cell-intrinsic defects in developing precursors.

In Chapter 3 of this thesis I examine genetic influences on age-related defects in murine B cell development and correlations between bone marrow B cell subsets and peripheral T cell subsets. It was known that longevity and age-related defects in T cell subsets are influenced by genetic differences between strains of inbred mice. The impact of genetic polymorphisms on age-related defects in B cell development had not been previously assessed. Nor was it known if these defects were correlated with age-related changes in peripheral T cell subsets. Here I present evidence that B cell subsets in the bone marrow are influenced by genetic polymorphisms between mice strains. Genetic

polymorphisms on Chromosomes 15 and 19 were found to influence the frequency of re-circulated and pre-B cells in the bone marrow of aged mice. Frequencies of bone marrow B cell subsets were compared with peripheral T cell subsets. Interestingly, an association between the frequency of pre-B cells was not observed with either re-circulated B cells in the bone marrow nor peripheral T cell subsets. However the frequency of pre-B cells was inversely correlated with the frequency of $B220^{int}IgM^{+}$ cells, a subset that was found to correlate with more advanced age-related T cell defects. In addition, frequencies of re-circulated B cells in the bone marrow were found to be associated with less advanced age-related defects in peripheral T cell subsets.

These observations indicate that defects in B cell development, including reduced *rag2* expression and V(D)J recombinase activity, are the result of changes in the aged murine bone marrow microenvironment. In addition, a genetic polymorphism located on Chromosome 19 influences the frequency of pre-B cells in aged mice. Furthermore the frequencies of B cell precursors in aged mice are not correlated with peripheral T cell subsets, but are correlated with frequencies of $B220^{int}IgM^{+}$ cells in the bone marrow. These observations advance our understanding of age-related defects in murine B cell development and may lead to identification of genes that influence B cell development in aged mice and humans as well as to help devise therapeutics aimed at restoring humoral immunity in aged individuals.

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CHAPTER I

Introduction

1.1 Age-related changes in the human immune system

With age, the human immune system is attenuated, resulting in increased susceptibility to pathogens and reduced tumor surveillance (1, 2). Vaccines have reduced efficacy in aged as compared to young individuals, memory responses do not last as long and there is reduced generation of high-affinity antibodies (2). Vaccines to hepatitis and influenza both have reduced protective response in the elderly. This immunosenescence is characterized by defects in both humoral and cellular immunity.

Some of the humoral (B cell related) changes are reduced antibody diversity, class switching and affinity maturation. Specific antibody titers in response to several vaccines are reduced in aged humans (1). Antibody responses to tetanus toxin, influenza, encephalitis viruses, salmonella, and pneumococcus bacteria are all reduced with age (3). Another age-related change in humoral immunity is an increase in low-affinity auto-reactive IgM antibodies in the serum (1, 2).

The peripheral B cell pool provides the ability to respond to new and previous encountered pathogens. This population is not static; cells are continuously removed and replaced by newly generated B cells throughout life. This process provides a constant source of diverse immunoglobulin (Ig) that enables the host to respond to new pathogens. A reduction in B cell development would impair the ability of the peripheral pool to respond to novel antigenic threats due to diminished introduction of diverse Igs. It has been suggested that in aged humans, generation of B cells in the bone marrow is greatly reduced (1). However, as addressed in the discussion section (Chapter 4) studies to determine if B cell development is limited in aged humans are inconclusive.

Of the human age-associated changes in cellular immunity, one of the most noted is a reduction in cellularity and output of the thymus, the primary organ for T cell development (2). In addition to reduced numbers, T cells in aged humans are also shifted from a naïve to memory phenotype. There is also an increase in anergic T cells and a greater frequency of T cells with signal transduction defects (4) (5). Proliferation and IL-2 production by T cells from aged humans are reduced, limiting both activity of cytotoxic effectors and $CD4^+$ T cell help for B cells (2). These changes in T cell development and activation are thought to contribute to reduced tumor surveillance and increased incidence of cancer. In addition, these changes contribute to reduced responses to pathogens and vaccines by both T cells and B cells as cytokines and direct cell contact with T helper cells is essential for B cell class switching and affinity maturation.

1.2 Murine models to investigate immunity and aging

Mice provide a useful model for the study of age-related changes in immunity. One attractive feature of mice is that their lifespan is much shorter than humans and age-related changes in immunity can be observed as early as 18 months of age. In addition, inbred strains of mice can be used to study the effect of aging on a genetically consistent population. The use of inbred mice in controlled living environments also allows the study of age-related defects independent of variables in environment, diet, pathogenic exposure, genetics and other factors that can influence health. Also, transgenic, knock-in, knock-out and mice with spontaneous gene mutations can be utilized to understand the impact of specific genes on age-related immune defects. One particular mutant strain of mice, the Klotho mice, displays premature aspects of aging including reduced B cell development and numbers of mature peripheral B cells (6). The number and frequency of pro-B cells and cells in further stages of B cell development are greatly reduced compared to wild-type mice. The defect is not cell-intrinsic as hematopoietic stem cells do not display reduced capacity of B lymphopoiesis either *in vivo* following adoptive transfer to wild type mice or *in vitro* during culture on stromal cells established from

wild-type mice (6). Thus the Klotho defect in B cell development is due to changes in the bone marrow microenvironment.

Aging characteristics in the murine immune system are also being studied to determine if genetic differences between strains contributes to the onset and severity of age-related phenotypes. A Quantitative Trait Locus (QTL) analysis has been used to identify polymorphisms that influence longevity and age-associated changes in immune status in aged mice (7, 8). In Chapter 3, I present and discuss results from a QTL analysis of B cell development in aged mice.

1.3 Age-related changes in the murine immune system

Like humans, mice display several age-associated changes in immune function, including reduced B and T cell generation (2, 9) and increased susceptibility to pathogens (e.g. influenza) (10).

Many of the T cell-specific alterations seen in humans also occur in mice. As in humans, the murine thymus undergoes involution with age. In addition, the percentage of T cells that are of the memory phenotype increases with age (2). Increased frequency of T cells with signaling defects (11, 12) and reduced cytokine production (2) by T cells from aged mice may contribute to reduced B cell activation and antibody response to vaccines and pathogens. TCR repertoire diversity is reduced in aged mice. CD8+ T cells express a wide range of TCR V_β regions is in young mice, however in aged mice there is restricted utilization of V_β gene segments with over-representation of some V_βs (indicating expansion of T cell clones) (13).

Several of the human age-associated defects in B cells are also observed in mice. This includes reduced generation of high-affinity antibodies in response to vaccination and infection (10), and loss of hypermutation, affinity maturation and germinal center formation (14). In addition, different V_H gene segments are used in aged as compared to

young mice in response to specific antigens (2, 15). In aged mice, the number of mature peripheral B cells is similar to that of young mice, however, the rate of turn-over is lower as fewer mature B cells are generated (9). The age-associated decline in humoral immunity may be partially due to reduced B cell receptor diversity and increased clonal expansion of limited numbers of B cells. These in turn could be the result of reduced generation of B cells in aged mice.

1.4 Murine B cell development

B cell development occurs continuously during life. In adult mice, this process occurs in the bone marrow and proceeds through an ordered series of developmental stages, concluding predominantly in the spleen. In the earliest stages, Multi-potent Progenitors (MPPs) undergo commitment to the lymphoid lineage(s) and become Common Lymphoid Progenitors (CLP) that can develop into B or T lymphocytes, Natural Killer (NK) cells or dendritic cells (16, 17). CLPs that commit to the B lineage then progress through stages of pro-B, pre-B and immature B cells in the bone marrow, and are then exported to the spleen where they progress through stages of immature transitional B cells and develop into mature naïve B cells.

Stages of B cell development are defined by acquired characteristics, such as the expression of lineage-specific genes and cell surface antigens (18). Differences in surface antigen expression define the B cell developmental pathway. Developing B cells are divided into pro-B ($IgM^{-}B220^{+}CD43^{+}$), pre-B ($IgM^{-}B220^{+}CD43^{-}$) and immature ($IgM^{+}B220^{LO}CD43^{-}$) stages in the bone marrow. Pro-B cells can be further divided into developmental Fractions (Fr.) A, B and C based on expression of CD24 (Heat Stable Antigen or HSA) and BP-1 (18).

FIGURE 1. Murine B Cell Development

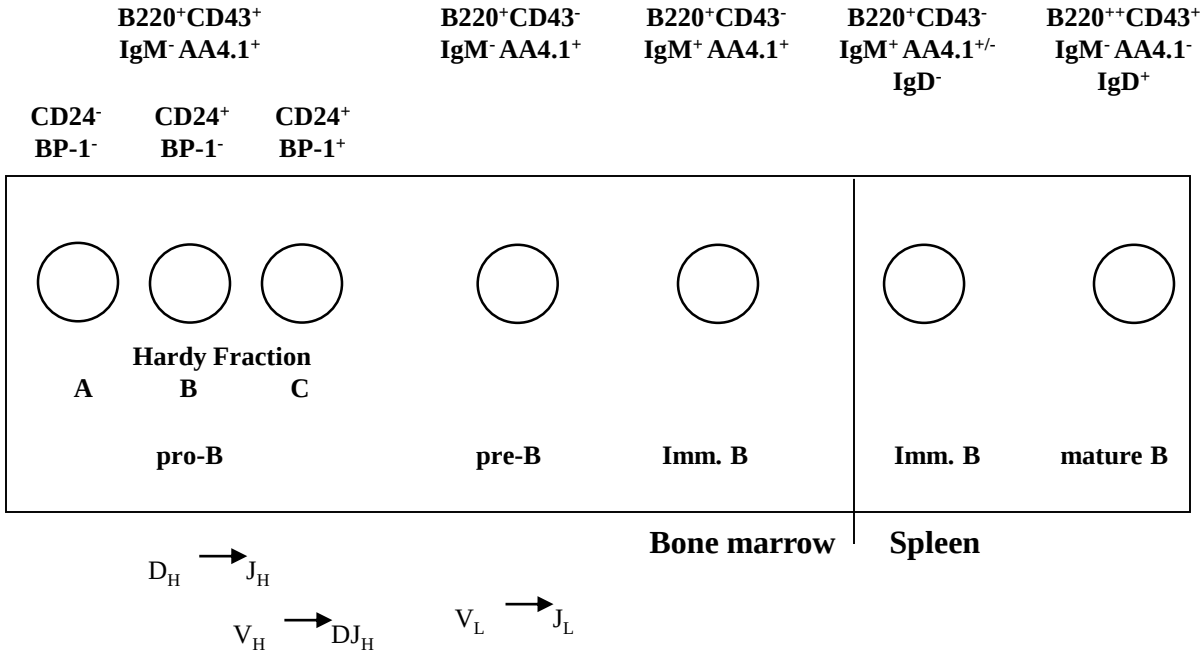


FIGURE 1. Murine B cell development. The schematic displays stages of B cell development in the bone marrow and spleen. Stage defining surface markers are displayed at the top. Recombination events are displayed at the bottom.

1.5 V(D)J recombination assembles complete Ig variable region genes during B cell development.

The variable regions of Ig genes are encoded by multiple Variable (V), Diversity (D), and Joining (J) gene segments located upstream of Ig Constant (C) regions. In mice there are 134 V_H, 13 D_H and 4 J_H gene segments in the immunoglobulin heavy chain (IgH) locus and 85 V_κ and 5 J_κ gene segments and 3 V_λ and 4 J_λ functional gene segments in the IgL chain loci (19). These gene segments are somatically rearranged to from functional Ig genes during B cell development by the process of V(D)J recombination (20, 21). In each B cell progenitor, a unique combination of V_H, D_H and J_H gene segments (one of each segment) are joined together to encode the IgH variable region, and one V_L and one J_L gene segment are joined to encode the IgL variable region. Each V, D, and J segment is flanked by conserved recombination signal sequence (RSS). The RSSs are evolutionary conserved sequences of DNA composed of heptamer and nonamer sequences that are generally conserved in content and are separated by either 12 or 23 base pair spacer (20, 21). The size of the RSS spacer mediates one level of regulation during V(D)J recombination; recombination events only occur between gene segments flanked by RSSs of different sizes, this is referred to as 12/23 rule.

Expression of Recombinase Activating Genes, *rag1* and *rag2* are essential to V(D)J recombination. *rag1* and *rag2* are coordinately expressed during B and T cell development (22-24). During V(D)J recombination, RAG1 and RAG2 bind to RSSs and assemble a synaptic complex with two Ig gene segments. Following formation of the complex, RAG1 and RAG2 introduce DNA double-strand breaks between the RSS and the flanking gene segment (25, 26). The DNA ends generated by RAG1 and RAG2 are referred to as the coding end for the segment containing the encoded Ig or TCR gene, and the signal end for the segment containing the RSS. After further coding end processing, the DNA breaks are repaired by non-homologous end joining to complete the recombination reaction. This requires activity of several ubiquitously expressed DNA double-strand break repair proteins: Ku70, Ku80, DNA-PK, XRCC4, DNA Ligase IV,

and Artemis. The two coding ends are ligated to form a coding joint, and the two signal ends are ligated to form a signal joint. In most Ig or TCR recombination events, the signal joint results in formation of circular piece of DNA containing the region that was originally between the two ligated gene segments, and this extra-chromosomal DNA is not replicated with cell divisions. As a result of V(D)J recombination, the coding joint now connects two gene segments that encode part of a new antigen receptor.

IgH and IgL genes are assembled during B cell development. During the pro-B cell stage of development, two recombination events in the IgH locus produce a rearranged, intact IgH variable region gene. First D_H to J_H recombination occurs, followed by V_H to D_HJ_H recombination. During the pre-B cell stage, a light chain gene is formed by one recombination event; V to J recombination of either Vκ to Jκ or Vλ to Jλ. Expression of *rag1* and *rag2* are up-regulated in the pro-B cell stage, when the IgH gene is assembled, and are again up-regulated in the pre-B cell stage when Ig light (IgL) chain gene is rearranged (22-24, 27). Regulation of *rag* expression during B cell development is discussed further below.

1.6 The pre-BCR checkpoint regulates B cell development.

Transit between the pro- and pre-B cell stages is regulated by signaling though the pre-B cell Receptor (pre-BCR). In the absence of pre-BCR signaling, cells fail to progress to the pre-B cell stage of development. The pre-BCR is composed of Igα and Igβ, the IgH chain and the surrogate light chain that is composed of λ5 and Vpre-B. Signaling through the pre-BCR is dependent upon the presence of a functional rearranged IgH gene. Production of functional IgH requires that following the recombination events, the variable region and constant region are in the same frame such that a protein can be correctly translated and that the protein can fold appropriately and associate with the surrogate light chain and Igα and Igβ. If the V_H to DJ_H joining on one allele does not produce a functional Ig heavy chain, pre-BCR signaling and progression to the pre-B cell

stage does not occur, thus providing time for V_H to DJ_H recombination on the other allele. If neither allele produces a functional heavy chain the pro-B cell undergoes apoptosis.

If a functional heavy chain is made, signals through the pre-BCR result in developmental changes in the precursor cell. Expression of *rag1* and *rag2* is turned off and the cell undergoes several rounds of proliferation. This accelerates the degradation of RAG1 and RAG2, as RAG2 is phosphorylated during the cell cycle resulting in ubiquitin-dependent degradation (21) and RAG1 half-life is shortened when not associated with RAG2 (21). This stops further recombination of IgH thus preventing rearrangement and expression of two IgH chains (a phenomenon known as allelic exclusion). In addition proliferation expands the number of precursors, resulting in greater numbers of pre-B cells than pro-B cells (3:1 ratio) in young mice.

Signaling through the pre-BCR induces several signaling pathways and changes gene expression in developing precursors. Igα and Igβ both contain immunoreceptor tyrosine-based activation motifs (ITAMs) that are phosphorylated upon pre-BCR assembly and provide sites for interaction with Syk tyrosine kinase (28). The adaptor protein BLNK (SLP-65, BASH) is phosphorylated at multiple N-terminal tyrosines upon interaction with Syk (29). BLNK then links pre-BCR signaling to several downstream signaling pathways by providing docking sites in the pre-BCR signal complex for SH2 domain-containing proteins that include PLCγ2, Btk, Vav and Nck (29). Mutations in BLNK result in a block in B cell development at the pro- to pre-B cell transition in both humans (30) and mice (31, 32).

Btk (Bruton's tyrosine kinase), a member of the Tec family of cytoplasmic protein kinases, transduces signals from the pre-BCR (33) (and the BCR). Humans with mutations in *btk* have an almost complete arrest in B cell development at the pre-B cell stage and an almost complete absence of mature B cells and circulating antibodies (34-36). Pre-B cell numbers are reduced in *xid* mice (mice in which the BTK gene is

defective) and *btk* knock-out mice as a result of reduced transition of cell from the pro- to pre-B cell stage of development (37-39). In addition, compared to wild-type mice, large-cycling pre-B cells from *btk*-deficient mice displayed enhanced proliferation and reduced progression into $CD2^{+}CD25^{+}$ surrogate $light^{-}$ small pre-B cells (37). BLNK also recruits Vav and Nck, both of which are important in cytoskeleton remodeling (40). Src-family tyrosine kinases (Blk, Fyn, Lyn) are also activated by pre-BCR signaling resulting in activation of NF-κB which promotes cell survival (40, 41). Gene expression assays have shown that pre-BCR signaling results in activation of 56 genes and repression of 34 genes (42).

As mentioned above, the pro- to pre-B cell checkpoint is dependent upon signaling through the pre-BCR. Defects in any of the pre-BCR components result in a lack of signaling and progression to the pre-B cell stage. For example, in the absence of a functional IgH chain, pre-BCR signaling does not occur and pro-B cells fail to progress to the pre-B cell stage. Expression of both *rag1* and *rag2* are essential to V(D)J recombination; therefore, in their absence an intact IgH chain is not produced and B cell development is blocked at the Fr. B/C pro-B cell stage (43, 44).

1.7 Murine B cell development is dependent upon factors from microenvironment.

Contact-mediated signals from bone marrow stromal cells and soluble factors are required for commitment, development, proliferation and survival of developing B cell precursors. Some of these effects are mediated by transcription factors that govern gene expression while some produce more direct changes through the activation of specific proteins. Pro-B cells are dependent upon direct contact with stromal cells while pre-B cells require soluble factors in the bone marrow environment.

IL-7 is produced by stromal cells and is essential for proliferation and differentiation of B cell progenitors *in vivo* (45, 46). The IL-7 receptor (IL-7R) is heterodimeric, composed of IL-7α and the γc chain which is utilized by IL-2, IL-4, IL-7, IL-9 and IL-15

(46, 47). IL-7α is first expressed in CLPs (16). Signaling through IL-7R is necessary for commitment to the B cell lineage as: 1) when cultured on stromal cells with IL-7, CLPs differentiate into B lineage precursors; 2) when CLPs were cultured in fetal thymic organ culture supplemented with IL-7 for 4 days they were unable to give rise to T cell precursors following intrathymic transfer, and 3) IL-7R$^{-/-}$ mice have normal frequencies of CLPs but a block in production of pre-pro-B cells and further B cell development in the bone marrow (48). In addition to lineage commitment in CLPs, signaling via the IL-7R during the pro-B cells stage of development results in hyperacetylation of histones in the 5' V_H regions (this is thought to result in accessibility to the V(D)J recombinase [discussed below]) (49). IL-7R signaling also results in proliferation and survival of pro-B cells.

The stromal cell product Stromal Derived Factor (SDF-1), also known as CXCL12, is a chemokine produced by bone marrow stromal cells that is essential for retaining B cell precursors in the bone marrow (50, 51). Incubation of B cell lines with SDF-1 results in activation of Rap1 and Rap2, Ras superfamily GTPases thought to regulate cell migration (52). Blocking activation of Rap1 and Rap2 results in decreased ability of B cell lines to migrate towards medium containing SDF-1 in a transwell assay (52). During B cell development interaction between SDF-1 and the receptor CXCR4 are important at the Lin$^-$CD19$^-$c-kit$^+$IL-7R□$^+$AA4.1$^+$ stage of fetal B cell development (53). Deletion of either SDF-1 or CXCR4 is embryonic lethal and these mice have very few pro-B cells in fetal liver at day 18 (54). Signaling through CXCR4 in pre-B cells results in proliferation of pre-B cells (54).

Another receptor/ligand pair that is critical for B cell development is fms-like tyrosine kinase-3 (flt3 or flk2) and FL (flt3 ligand). Flt3 is expressed in developing precursors through the pre-B cell stage of development (55). FL deficient mice have reduced numbers of CLPs (56) and flk2-/- mice have reduced numbers of pro-B cells (57). FL and IL-7 are sufficient and required for stromal-independent *in vitro* B cell

development from multi-potent hematopoietic progenitors (58) and $FL^{-/-}IL\text{-}7R^{-/-}$ double deficient mice completely lack conventional IgM^{+} B cells (59).

Signals from the bone marrow microenvironment result in activation of transcription factors at different stages of B cell development. In turn, transcription factors activate and suppress genes that establish and enforce lineage commitment, promote survival, proliferation and development.

1.8 Murine B cell development is regulated by transcription factors.

Graded expression of the transcription factor PU.1 (a member of the ets protein family) in multi-potential progenitors regulates commitment to the myloid, dendritic, and B lineages (60, 61). Low-level expression of PU.1 promotes B cell development (60, 61). In the absence of PU.1, development of T lineage cells but not B or myeloid cells can occur (41, 61). The ability of PU.1 to direct B lineage commitment, development and proliferation is through the regulation of *IL-7Rα* transcription (62). IL-7Rα signaling is essential for commitment of progenitors to the B lineage (48). Retroviral transduction of IL-7Rα into PU.1-/- progenitors restores proliferation in response to stromal cells and IL-7 and results in a low frequency of precursors that develop into CD19 pro-B cells that express *mb-1* (encodes Igα*)*, *B29* (encodes Igβ*)*, *VpreB*, *λ5*, *EBF* and *pax5* (62). This does not indicate that PU.1 activates these genes, rather that PU.1 through expression of IL-7Rα supports proliferation and differentiation to the pro-B stage of development where transcription factors *E2A*, *pax5* and *EBF* are expressed.

Pax5 (BSAP or B cell-Specific Activator Protein), E2A and EBF (Early B Cell Factor) are transcription factors whose function is essential in precursors committed to the B lineage (63). Pax5 maintains B lineage commitment by suppressing expression of NOTCH, which can promote T cell development over B cell development (41). Following experimentally-induced loss of *pax5*, pro-B cells can revert to macrophages and T cells (64). *pax5* regulates expression of genes required for B cell development,

including *blnk* (SLP-65) and *CD19*. *pax5* may be regulated by E2A and EBF (discussed below), and in conjunction these three factors appear to regulate expression of *mb-1* (encodes Igα*)*, *B29* (encodes Igβ*)*, *VpreB*, *λ* (41, 61). *pax5*-deficient mice have an essentially complete block in B cell development at the pro-B cell stage (at Hardy Fr. B) and while cells have DJ_H junctions, V to DJ_H are almost undetectable (65-67).

E2A and EBF (Early B Cell Factor) also regulate B cell development by activating B cell-specific genes. *E2A* encodes proteins E12 and E47, which are critical for B cell development. Mice lacking *E2A* have a block in B cell development prior to Ig gene rearrangement (41, 68-70). E47 was recently found to bind conserved E-boxes in a regulatory element for RAG expression (*ERAG*) (explained below) (71). Ectopic expression of E12 up-regulates expression of EBF (69). Deletion of EBF results in arrested B cell development prior to Ig gene rearrangement and a lack of Pax5 expression (63). Binding sites for EBF have been detected in *λ5, VpreB*, and *Igβ* (63) and EBF may regulate *pax5* (69). Together *E2A, EBF* and *pax5* control transcription of the early B cell genes *mb-1* (encodes Igα*)*, *B29* (encodes Igβ*)*, *VpreB*, and *λ5* (41, 61). E2A and EBF also regulate accessibility of the recombinase to the Ig loci as transfection of *E2A*, *EBF*, *rag1* and *rag2* into non-lymphoid cells results in V(D)J rearrangement (72, 73). Overexpression of *E2A*, *rag1* and *rag2* in a human cell line resulted in Ig V_κ1J but not Igλ recombination. Expression of *EBF*, *rag1* and *rag2* resulted in V_λIII-J$_\lambda$1 recombination. When *E2A*, *EBF*, *rag1* and *rag2* were expressed together, D_H4J recombination occurred but V_H to DJ_H was not observed (72, 73).

There are two requirements for V(D)J recombination: accessibility of Ig gene segments for recombination and the enzymatic activity of the V(D)J recombinase. Both accessibility and recombinase activity are developmentally regulated

1.9 Expression of *rag1* and *rag2* are developmentally regulated.

Highly conserved core promoters and cis-elements control transcriptional regulation of *rag1* and *rag2*. *rag1* and *rag2* are located in one locus in the genome of all animals studied and are convergently transcribed (21, 74). The murine and human promoters for both *rag1* and *rag2* are highly conserved (75). *rag1* and *rag2* are the only essential lymphoid-specific components of the V(D)J recombinase and their expression is tissue and developmental stage-specific. While murine and human *rag1* expression can be observed in non-lymphoid cell types, *rag2* expression is lymphoid specific and differentially regulated in B and T cells (75). *pax5* is expressed in B but not T cells (76) and binds to a conserved sequence essential to expression of *rag2* (75). *Myb* is expressed in developing T but not B cells (77, 78), binds the *rag2* promoter and is critical for *rag2* promoter activity in T cells (79).

In addition to core promoters, cis-acting elements 5' of *rag2* are essential to expression of both *rag1* and *rag2* and contribute to differential regulation in B and T cells (80, 81). The *ERAG* element located 22 kb 5' of *rag2* has 90% DNA sequence homology between mice and humans and is essential to *rag1* and *rag2* expression in B but not T lineage cells (71). *ERAG*$^{-/-}$ mice have 3-fold fewer pre-B cells and reduced numbers of mature splenic B cells compared to wild type mice. In *ERAG*$^{-/-}$ mice transcription of *rag1* and *rag2* are reduced in pro-B cells (5-15 fold and 2-3 fold), as is frequency of dsDNA breaks associated with V to DJ_H joining (71). Recent work has shown that the V(D)J recombinase is active in the common lymphoid progenitor (CLP) and is largely controlled by the *ERAG* enhancer in the B lineage. This observation supports the concept that a B lineage-specific transcription program is already established in the CLP (82). E2A binds *ERAG* in pro-B cells as shown by chromatin immunoprecipitation (ChIP) assay and transfection of E2A into 293T cells induces expression of *rag1* and *rag2* (71). It is thought that additional uncharacterized cis-acting elements exist upstream of *rag2* as there are approximately 20 non-coding sequences of 200 bp or more that lack significant ORFs and have 80% homology between mouse and human (21).

1.10 Regulation of accessibility to Ig gene segments

The same recombinase rearranges both Ig and TCR gene segments; however, recombination events occur in a tissue and stage-specific manner. IgH chains are only completely recombined in pro-B, IgL (κ and λ) in pre-B, TCRβ in pro-T and (most) TCRα in pre-T cells). It has been proposed that selective rearrangement of Ig loci is governed by accessibility of the recombinase to Ig gene segments (83). This model is supported by the observation that in *in vitro* assays, RSSs in DNA harvested from non-lymphoid cells are inaccessible to RAG-mediated cleavage; however chromatin from lymphocytes is accessible in a pattern that matches Ig recombination in the cell type from which it was purified (21).

Regulation of Ig gene segment accessibility is not fully understood and may be linked to transcriptional accessibility. The transcriptional promoters and enhancers are required for efficient cleavage by the recombinase (21). Signaling through the pre-BCR results in binding of transcription factors to the Igκ enhancer and activation of germ-line Igκ transcription (21). V_κ to J_κ rearrangements is absent in mice in which the two κ locus enhancers have been deleted (20, 84). Deletion of the T early α promoter (TEA) (located 5' of the J_α segments) results in reduced rearrangements of the most 5' J_α segments located 15 kb downstream of the promoter (20, 85). It is thought that promoters and enhancers allow accessibility to DNA in a localized manner; however there are examples of transcribed Ig gene segments that do not undergo V(D)J recombination and examples of V(D)J recombination in the absence of germ-line transcription (20).

We are just beginning to understand how accessibility of Ig gene segments for transcription or recombinase activity is regulated by DNA methylation, histone modification and nuclear localization (21). Methylation of CpG islands leads to gene inactivity, and less susceptibility to recombinase cleavage (20, 21). Demethlylation of the Igκ locus correlates with active recombinase (20, 21).

Accessible DNA is characterized by hyper-acetylated lysine residues in the N-terminus of histones H3 and H4 (20). The histones associated with the V_H region in pro-B cells undergo histone acetylation after IL-7 stimulation (86). This acetylation of V_H regions occurs prior to V_H to DJ_H recombination (86, 87). Expression of transmembrane mu in the pre-BCR results in de-acetylation of the V_H locus and increased acetylation of the κ locus (87). It was also observed that the chromatin including the Ig gene segments in the IgH locus are acetylated but the intervening stretches of DNA are not, indicating a specific regulatory mechanism that specifically provides access to gene segments (87). Further evidence of the role of histone acetylation in control of V(D)J recombinase accessibility comes from recent investigation of the role of *Ezh2*. Deletion of *Ezh2*, a factor known to regulate chromatin structure, resulted in lower histone methylation of 5' V_H gene segments (specifically the J558 family) in pro-B cells following incubation with IL-7 (88). In addition, pro-B cells from $Ezh2^{-/-}$ mice have diminished V to DJ_H rearrangement and reduced number and frequency of pre- and immature B cells (21, 88). Nuclear localization of gene segments also contributes to regulation of recombinase accessibility. Transcribed and non-transcribed regions of DNA are located in different domains of the nucleus. In non-lymphoid cells and T cells, the IgH alleles are located in peripheral nuclear areas; however in pro-B cells they were localized in central nuclear area (89).

1.11 B cell development is attenuated in aged mice.

Frequencies of B cell subsets in the bone marrow and B cell generation undergo age-associated changes in mice. In aged mice B cell generation is markedly reduced (9, 90). Fewer mature B cells are produced but the number of mature B cells is similar to that of young mice due to a significant increase in the half-life of mature cells (1, 9). Frequencies and numbers of B cell subsets in the bone marrow also change with age. The most noted change is the reduced frequency and number of pre-B cells. The extent of reduction, and age at which this occurs varies between reports (90-95). Initial reports indicated that numbers of pro-B are not reduced in aged mice. However, our results

(Chapter 2), along with two recent reports indicate that numbers of pro-B cells are significantly reduced in aged mice (48, 91). This apparent discrepancy is addressed in Chapter 2. In addition, the number of mature re-circulated B cells in the bone marrow increases in aged mice (9, 91).

The onset and severity of reduced pre-B cell numbers can vary substantially between mice, even of the same age, strain, gender and environmental conditions. Mice with more extensive loss of pre-B cells tend to have reduced numbers of pro-B cells while mice with moderate reductions in pre-B cells tend to have numbers of pro-B cells that are slightly reduced, or similar, to that seen in young mice (91). Numbers of mature re-circulated B cells in the bone marrow of aged mice are higher in mice with moderate reduction in numbers of pre-B cells, but are reduced in mice with severe reductions in numbers of pre-B cells (91). These defects may be due to one underlying mechanism that affects B cell development at more than one stage or due to multiple distinct changes that occur with age.

1.12 Is pre-B cell generation reduced in aged mice?

A primary goal of this thesis is to understand mechanism(s) that contribute to the reduction in numbers of pre-B cells in aged mice. Some potential mechanisms include: 1) increased apoptosis of pre-B cells, 2) reduced transit times of pre-B cells to the immature B cell compartment, and 3) reduced generation of pre-B cells. It is formally possible that each mechanism contributes to the reduced numbers of pre-B cells.

Reduced generation of pre-B cells is supported by the observation that development within pro-B cells is impaired in aged mice. Two recent papers and my data in Chapter 2 find a reduction in numbers of cells in more mature pro-B cell stages (91, 95, 96). In addition, *rag1* and *rag2* mRNA levels decline dramatically when measured in the total bone marrow of aged mice (3, 97, 98). RAG1 and RAG2 are essential to the generation of pre-B cells. This reduction in expression of *rag1* and *rag2* could suggest attenuated

pre-B cell generation in aged mice. This idea is one of the central hypotheses of this thesis: that in aged mice reduced *rag* expression in pro-B cells results in reduced V(D)J recombinase activity and in turn, reduced generation of pre-B cells. While decreased levels of *rag* expression within the bone marrow of aged mice was previously demonstrated (97, 99), the effect of age on *rag1* and *rag2* expression within pro-B cells had not been directly demonstrated before this work. In addition, it was not known if the age-related decline in *rag* expression is due to a global loss of bone marrow cellularity independent of regulation of *rag* expression, a decrease in the number of specific cells that express *rag* or a decline in the per cell level of *rag* expression within pro-B cells. BRDU labeling experiments have shown that generation of pre-B cells is reduced in aged mice as compared to younger mice and that the transit time for pre-B cells is similar in aged vs. young mice (Dr. M. Cancro, UPENN, personal communication; addressed in Chapter 4 Discussion.)

Another possible mechanism that would contribute to reduced numbers of pre-B cells is decreased signaling through the pre-BCR due to reduced expression of λ5 (95). λ5 is a component of the pre-BCR that is essential for developmental progression to the pro-B cell stage. Expression of surface and cytoplasmic λ5 is reduced in pro-B cells of aged mice (95).

1.13 Microenvironment or cellular defects underlie reduced B cell development with age.

The decrease in numbers of pre-B cells in aged mice could be due to either cell-intrinsic defects in the B cell precursors themselves or alterations in the developmental microenvironment. The bone marrow microenvironment is thought to provide essential signals for stimulating expression of *rag* during B cell development: however, environmental and cell-intrinsic causes for reduced numbers of pre-B cells in aged mice had not been addressed prior to this work.

Pre-B cell numbers decline with age, and thymic involution progresses with age. It has been proposed that reduced thymic output affects the bone marrow microenvironment and contributes to reduced numbers of pre-B cells in aged mice (98). This is supported by the observation that numbers of pre-B cells and expression of both *rag1* and *rag2* are lower in young athymic mice as compared to young wild-type mice (97, 98). In addition, injection of T cells or supernatant from cultures of activated T cells into nude mice resulted in increased expression of *rag1* and *rag2* in bone marrow of athymic mice (97, 98). These observations have led to the hypothesis that *rag1* and *rag2* expression in developing B cells is influenced by T cell factors, which are absent in nude mice and decline with thymic involution during aging. Additional support for the hypothesis that the decline in B cell development with age is due to defects in the microenvironment comes from stromal cell culture experiments. Stromal cell cultures established from aged mice are less supportive of proliferation and development of pro-B cells than cultures established from younger mice (94). IL-7 is critical for B cell development. When an IL-7-dependent cell line was cultured on stromal cells from young and aged mice, the percent of cells in cell cycle was higher in stromal cultures established from young mice at 25, 50 and 75 hours (93). This indicates that availability of IL-7 may be limited in stromal cells from aged mice.

Reduced response to IL-7 could also be due to cell-intrinsic defects in precursors from aged mice. Pro-B cells from aged mice proliferate to a lesser extent than pro-B cells from young mice when cultured *in vitro* with IL-7 (93, 96, 100). Interestingly, expression of IL-7R is similar between precursors from young and aged mice (94, 96, 101). This could indicate that intrinsic signaling in response to IL-7 is defective in B cell precursors in aged mice. Most of the evidence supporting cell-intrinsic, age-related defects is from *in vitro* experiments. It is possible that the cellular defects observed in culture are due to changes that are "imprinted" on developing B cells by changes in the *in vivo* microenvironment. Developing cells may go through stages where lack of exposure to various stimuli results in developmental arrest that the cells cannot recover from. This

could result in the presence of pro-B cells that have impaired function but have not yet been removed from the bone marrow by apoptosis. Another important goal of this thesis work was to determine if age-related loss of pre-B cells and reduced expression of *rag2* are the result of cell-intrinsic defects or defects in the bone marrow microenvironment.

1.14 There are numerous unanswered questions regarding B cell development and aging.

B cell development is reduced in aged mice and is characterized by a severe reduction in numbers of pre-B cells (90, 95, 96, 98, 101, 102). In addition, expression of both *rag1* and *rag2* are reduced in the bone marrow of aged mice. However, before this work, it was not known if *rag* expression was reduced in pro-B cells. And it was not known if a reduction in *rag* expression results from a reduction in expression in the percent of pro-B cells that express *rag* or due to reduced per cell expression (or both). In addition, until this work, it was not known if the drop in *rag* expression is consistent with a decrease in V(D)J recombinase activity. It was possible that *rag* expression levels decline but are still sufficient for effective V(D)J recombination. Furthermore, it was unknown as to whether reduced *rag* expression contributes to loss of pre-B cells in aged mice. In addition, it was not known if loss of pre-B cells in aged mice was the result of defects in the bone marrow microenvironment or cell intrinsic defects. These questions are addressed in Chapter 2.

Another area that had not been addressed is the impact that genetics has on age-associated defects in B cell development. It is possible that the impact of age-associated defects in B cell development and immunity are regulated by specific genes, and that certain alleles provide increased or reduced susceptibility to age-related defects. The identification of these genes could eventually lead to new therapeutics to restore generation of mature B cells in the aged.

Furthermore, the relationships between B cell development and other lymphocyte populations in aging have not been explored. In aged mice, there are reduced numbers of pro- and pre-B cells, and an increase in the number of re-circulated mature B cells in the bone marrow. In addition, reduction in size and output by the thymus occurs with age and there is an increase in anergic and memory peripheral T cells (103). It is possible that these changes in peripheral T cell subsets contribute to altered B cell development. Or it may be that age-associated changes in B and T cells are the result of a common underlying mechanism. It is also possible that B and T cell changes occur independent of each other and result from independent mechanisms. These questions are addressed in Chapter 3.

1.15 Approach and summary of results.

Expression of *rag2* is lower in pro-B cells of aged mice.

In this thesis I have compared expression of *rag2* and V(D)J recombinase activity at the single-cell level in pro-B cells from young and aged mice (Chapter 2),. To characterize expression of *rag2,* I used two different mouse strains that contain GFP as reporters of *rag2* expression (discussed below). As *rag1* and *rag2* are co-ordinately regulated measurement of *rag2* expression is expected to serve as an indicator of expression of both genes.

The GFP transgenic *rag2* reporter (NG strain) was created in the lab of Dr. Michel Nussenzweig (80). In this system, GFP serves as a reporter of *rag2* gene expression. The transgene contains 200 kb of the *rag* genomic locus, with GFP inserted in place of the major *rag2* exon. The mice retain normal RAG2 function because the endogenous *rag2* locus remains intact. In addition, the transgene does not affect the development or health of mice. The GFP in this transgene is an accurate and sensitive reporter of *rag2* transcription levels and enzymatic activity (80).

The second model used in this work is a RAG2-GFP fusion protein knock-in (KI) mouse made in the lab of Dr. Fred Alt (81). These KI mice are accurate reporters of

RAG2 levels in single cells, and have two important advantages compared to NG mice: 1) the GFP reporter is located within the endogenous *rag2* locus, and 2) GFP is expressed as a fusion protein with RAG2 and thus serves as a direct reporter of cellular RAG2 protein levels. In these mice, the RAG2-GFP fusion protein maintains normal RAG2 enzymatic activity and B cell development is not altered.

Through FACS analysis of GFP fluorescence, the expression of *rag2* can be assessed at the single-cell level in conjunction with surface markers used to define distinct stages of B cell development. This provides two advantages over previously used methods: 1) measurement of the level of *rag2* expression in live, single cells rather than lysates of bulk cell populations, and 2) assessment of *rag2* expression within pro-B cells. Using these systems I was able to directly analyze *rag2* expression at the single cell level and determine that *rag2* expression is reduced in pro-B cells of aged mice. Reduced *rag2* expression in pro-B cells of aged mice is due to expression in fewer cells, as the pro-B cell that express rag2 have "per cell" levels that are comparable between young and age mice.

1.16 Reduced expression of *rag2* is consistent with reduced recombinase activity in pro-B cells and reduced numbers of pre-B cells.

In this work, an assessment of V(D)J recombinase activity at the single cell level in pro-B cells was also conducted to determine if the loss of *rag2* expression in aged mice was consistent with a loss of V(D)J recombinase activity. This was done using H2-SVEX V(D)J recombination substrate transgenic mice created in our lab. In this transgenic mouse VEX, a spectrally-distinct form of GFP, (104) serves as a reporter of V(D)J recombinase activity. Expression of the H2-SVEX transgene is driven by the MHC class I promoter (active throughout all hematopoietic cells), and is therefore independent of B-cell-specific transcription regulation (82). Cellular GFP fluorescence is acquired following recombination of the transgene and is dependent upon sufficient expression and activity of all factors required for V(D)J recombination. Using H2-SVEX

x NG double transgenic mice, I measured *rag2* expression and V(D)J recombinase activity to determine if the aged-related decrease in *rag2* expression results in decreased V(D)J recombinase activity and generation of pre-B cells. I found that V(D)J recombinase activity is reduced in pro-B cells of aged mice, and correlated with the reduction in *rag2* expression. Furthermore, the losses of *rag2* expression and V(D)J recombinase activity in aged mice were associated with reduced numbers of pre-B cells. Aged mice with the most severe loss of *rag2* expression and V(D)J recombinase activity also had the most severe loss of pre-B cells. In other mice, loss of *rag2* expression was minimal compared to young mice, and recombinase activity and numbers of pre-B cells were only marginally decreased. These results are consistent with the hypothesis that the decline in pre-B cell numbers in aged mice is due to reduced *rag2* expression in pro-B cells.

1.17 Age-associated defects in the bone marrow microenvironment are sufficient to produce reduced *rag2* expression and V(D)J recombinase activity in pro-B cells, and reduced numbers of pre-B cells.

I conducted reciprocal adoptive transfer experiments to determine if reduced *rag2* expression and V(D)J recombinase activity in pro-B cells, and reduced numbers of pre-B cells are due to defects in the bone marrow microenvironment or the results of cell-intrinsic defects. Adoptive transfer of whole bone marrow from young mice into mice of various ages was used to assess defects due to the aged microenvironment. Donor mice were NG x H2-SVEX double transgenic mice. This provided the ability to measure both *rag2* expression and V(D)J recombinase activity in pro-B cells and compare numbers of pre-B cells. Using this system, I determined that age-associated changes in the hematopoietic microenvironment are sufficient to produce reduced *rag2* expression, V(D)J recombinase activity and pre-B cells (Chapter 2).

To specifically determine if the loss of pre-B cells is influenced by cell-intrinsic defects in aged mice, I conducted adoptive transfers from aged and young mice into

young recipient mice. Donor mice were aged NG transgenic mice that had notable defects in *rag2* expression in pro-B cells and reduced numbers of pre-B cells. Recipient mice were young, wild-type mice. In Chapter 2, I show that bone marrow from aged mice is not intrinsically defective in regards to ability to express *rag2* in the pro-B cell stage and generate numbers of pre-B cells.

1.18 Frequencies of pre-B cell and re-circulating B cell in the bone marrow of aged mice are influenced by specific genetic polymorphisms.

In order to determine if genetic differences between mouse strains contribute to the age-related loss of pre-B cells, I conducted a Quantitative Trait Locus (QTL) analysis in collaboration with Dr. Richard Miller (University of Michigan). This analysis compared 87 simple-sequence length polymorphisms (SSLPs), and B cell developmental subsets and peripheral T cell subsets in a genetically heterogeneous cohort of aged mice. This cohort (termed UM-HET3) was produced by mating BALB/c x C57BL/6 F1 females and C3H/HeJ x DBA/2J F1 males. These four strains were selected because they are spread wide apart in their ancestral lineage (105) and thus should maximize allelic variation within the cohort. In addition, information on genetic variations between these strains, including SSLPs, is available. This method has been successful in identifying polymorphisms related to longevity (8) and age-related T cell phenotypes (7). We expected this cohort to display diversity with respect to age-related alterations in B cell developmental subsets and thus enable us to determine if genetic polymorphisms between these strains are associated with the age-related changes in B cell development. In addition, frequencies of peripheral T cell subsets and T cell proliferation were compared with frequencies of B cell subsets to determine if correlations exist. These could suggest cause and effect relationships or common mechanisms.

In Chapter 3, I present evidence that the frequencies of pre-B cells and re-circulated B cells in aged mice are influenced by specific genetic loci. A polymorphism located on chromosome 19 is correlated with frequencies of pre-B cells and a polymorphism on

chromosome 15 is correlated with frequencies of re-circulated B cells in the bone marrow.

1.19 Analysis of relationships between B cell subsets in the bone marrow and peripheral T cell subsets.

I also wanted to determine if frequencies of B cell precursors in the bone marrow were associated with T cell subsets in the periphery. The aged cohort of UM-HET3 mice was used in this analysis. In Chapter 3, I show that the frequency of pre-B cells in aged mice is not correlated with frequencies of peripheral memory or anergic T cell subsets. However, the frequency of pre-B cells is inversely correlated with the frequency of $B220^{int}IgM^{+}$ cells in the bone marrow, and frequency of re-circulated B cells is associated with the frequency of peripheral anergic T cells (described in Chapter 3).

These observations contribute to our understanding of the age-related impact on B cell development in mice and suggest that therapeutics to restore B cell production in aged humans may come from better understanding of the changes in the bone marrow microenvironment.

CHAPTER II

Expression of *rag2* and V(D)J recombinase activity are reduced in pro-B cells of aged mice and are correlated with loss of pre-B cells.

INTRODUCTION

In both aged humans and aged mice, the adaptive immune response is severely compromised and characterized by decreased ability to respond to new pathogens (2, 106, 107) and less protective immunity following vaccination (108, 109). These changes are due, in part, to decreased production of B lymphocytes and loss of diversity of the immunoglobulin (Ig) repertoire (3, 9, 97-99, 110-113). However, the molecular mechanisms that underlie age-associated diminished B cell production remain unclear.

V(D)J recombination is essential to the production of lymphocytes as it assembles Ig and T-cell receptor genes during B and T cell development. Recombinase Activating Genes, *rag1* and *rag2,* are lymphocyte-lineage specific proteins that are coordinately expressed during B and T cell development (22-24). *rag1* and *rag2* are required to produce site-specific DNA double-strand breaks that initiate V(D)J recombination (22, 23). *rag1* and *rag2* gene expression is up-regulated in pro-B cells when the Ig heavy (IgH) chain is assembled, and in pre-B cells when the Ig light (IgL) chain is assembled (22, 23). The Ig heavy chain is an essential component of the pre-B cell receptor (pre-BCR), which transmits signals that enable progression from the pro- to pre-B cell stage of development (114, 115). In the absence of *rag1* or *rag2*, an intact IgH chain is not produced resulting in a complete arrest of B cell development at the pro-B cell stage (43, 44).

B cells are generated continuously during life (9). This process is impaired in aged mice; one specific change is fewer pre-B cells are found in bone marrow (3, 90, 91, 101).

There are several possible explanations for this: 1) reduced production of pre-B cells due to impaired development of pro-B cells, 2) decreased survival of pre-B cells or 3) an acceleration of the transit time between the pre-B and immature B stages of development. Reduced production is likely to be the major contributing factor based on several observations. Development within the pro-B cell population is impaired in aged mice, as indicated by reduced numbers of cells in the more mature pro-B cell stages and reduced expression of λ5 in pro-B cells (91, 95, 96). Also, recent kinetic analyses indicate young adult mice generate 9 to 13 million pre-B cells daily, whereas aged individuals produce only 2 to 5 million pre-B cells per day (personal communication, Dr. Michael Cancro [addressed in Chapter 4 Discussion]). Nonetheless, the renewal rates of pre-B cells are very similar in young and aged mice (32 to 42% per day), indicating that the residency time within the pre-B cell pool is unchanged in aged individuals (personal communication, Dr. Michael Cancro [addressed in Chapter 4 Discussion]). An age-associated reduction of *rag* expression in pro-B cells, and a resulting reduction in V(D)J recombinase activity may contribute to decreased size of the pre-B cell compartment in aged mice.

rag1 and *rag2* mRNA levels decline dramatically when measured in the total bone marrow of aged mice (3, 97, 98). However, it is unknown if this reduction is the result of diminished numbers of cells within the developmental stages where *rag1* and *rag2* are expressed, or reduced *rag* expression within those subsets. It is possible that the age-associated reduction in pre-B cells alone could account for the reduction in *rag* expression when measured in total bone marrow. This is supported by the observation that the reduction in pre-B cells is extensive in aged mice; whereas pre-B cells outnumber pro-B cells 3:1 in young mice, they are often equal to, or less than, the number of pro-B cells in aged mice (3, 90, 91, 101). Alternatively, reduced *rag1 and rag2* expression in aged mice may be due to a reduction in the frequency of pro- and pre-B cells that express *rag1* and *rag2*. *Rag1* and *rag2* expression levels are expected to determine and limit levels of V(D)J recombinase activity (82, 116-118); thus a reduction in *rag* expression

within these subsets could impair recombination. However, an assessment of *rag* expression levels or V(D)J recombinase activity in developing B cell precursors in aged mice has not been conducted.

It is unknown if the age-related reduction in B cell development is the result of changes in the bone marrow microenvironment or due to cell-intrinsic defects. Evidence for cell-intrinsic defects includes the observation that *in vitro* cultures supplemented with IL-7 drive less proliferation of sIg^- precursors and pro-B cells from aged mice versus young mice (90, 93, 100). Evidence for changes in the microenvironment include the finding that expression of both *rag1* and *rag2* in total bone marrow and numbers of pre-B cells are reduced in athymic mice (97, 98). In addition, stromal cell cultures established from aged mice support less *in vitro* proliferation of pro-B cells or pre-B cell lines than cultures established from young mice (93, 94, 101). Here, we ask whether *rag2* expression is decreased within pro-B cells of aged mice, and whether this decrease is concurrent with a reduction in V(D)J recombinase activity and thus may explain the decreased numbers of pre-B cells. To gain insight into *rag2* expression and its effect on regulation of V(D)J recombination during aging, we conducted flow cytometric analyses of bone marrow from the following young and aged mice: 1) transgenic mice that express a GFP reporter of *rag2* transcription (24), 2) knock-in mice that express GFP as a reporter of *rag2* transcription and RAG2 protein (81), and 3) double transgenic mice in which the *rag2* expression reporter system is paired with a transgenic reporter of V(D)J recombinase activity (82). This approach enabled us to determine that the lower pre-B cell number in aged mice is correlated with changes that occur in the pro-B cell stage of development and include both reduced expression of *rag2* and reduced V(D)J recombinase activity.

We also addressed if age-related defects in B cell development are the result of an altered bone marrow microenvironment or changes that are intrinsic to developing B cells. We distinguish the impact of the aged microenvironment and cell intrinsic defects

by using an adoptive transfer model. We found that alterations in the hematopoietic microenvironment of aged mice are sufficient to produce the reduction in *rag2* expression and V(D)J recombinase activity in pro-B cells and the reduced numbers of pre-B cells.

These results lead to the conclusion that defects in B cell development in aged mice occur as early as the pro-B cell stage of development and suggest that diminished expression of *rag2* and reduced V(D)J recombinase activity are associated with reduced numbers of pre-B cells. In addition, our observations indicate that defects in the aged bone marrow microenvironment are sufficient to result in reduced B cell development. This suggests that extrinsic factors in the bone marrow that decline with aging could be identified and lead to development of therapeutics for age-related defects in human B cell development.

MATERIALS AND METHODS

Mice

C57BL6J, CBA/J, FVBN/J and B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (CD45.1) were obtained from Jackson Laboratories (Bar Harbor, ME). NG transgenic mice on the FVBN background were obtained from Michel Nussenzweig (Rockefeller University, NY, NY) (24, 80). NG transgenic mice were mated to CBA/J wild type mice to create F1. NG mice were also crossed to C57BL6/J mice for six to nine generations, and then crossed to H2-SVEX mice and B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (CD45.1) mice. RAG2:GFP Knock-in mice on a mixed 129 x C57BL/6 background were provided by Fred Alt (Harvard Medical School, Boston, MA)(81). H2-SVEX mice (SB110 line) on the C57BL/6 background were constructed as described in Borghesi et al. (82). Aged C57BL/6 mice used as adoptive transfer recipients were obtained from either The National Institute of Aging Repository at Harlan Sprague Dawley (rederivation in 1998, from Jackson Laboratory stock) or from Dr. Raymond Welsh (Dept. of Pathology, UMMS; obtained from Jackson Laboratories (Bar Harbor, ME) at 2-4 months of age and housed in Specific Pathogen Free conditions). Unless indicated otherwise, all mice were maintained by the Department of Animal Medicine, UMMS.

Adoptive transfers

For adoptive transfers of bone marrow from young mice into young and aged recipient mice, bone marrow was harvested from leg bones of 3 - 12 week old NGxH2-SVEX double transgenic mice on the C57BL/6 CD45.1 background. Bone marrow from multiple donors was pooled, washed in Hanks' Balanced Salt Solution (Life Technologies, Rockville Maryland, Cat. 14025), and suspended to 10 million cells per ml. Young and aged recipient mice received lethal irradiation (850-1000 rads) 16-20 hours prior to adoptive transfer. Recipient mice received 3.5 or 6 million cells via tail vein injection. Neomycin sulfate (Sigma N-6386) and polymyxin B sulfate (Sigma P-4932) were administered through drinking water starting 4 days prior to irradiation and ending 14 days post adoptive transfer. Water bottles were changed every two days. Five to six weeks after adoptive transfer, bone marrow was harvested from recipient mice for

analysis. At this time point, pro- and pre-B cells detected in the recipient mice have presumably differentiated from hematopoietic stem cells or very early progenitors present in the transferred marrow (17, 119). Gross inspection of young and aged mice was performed prior to and after euthanasia. Mice displaying signs of tumors or other abnormalities were eliminated from analysis. In addition, mice were excluded from analysis if less than 30% of pro-B cells were of donor origin. This criterion resulted in exclusion of seven out of 37 mice as engraftment was efficient in most mice (11 out of 17 aged mice and all young mice had over 85% engraftment). Although generally efficient, engraftment varied between mice, and therefore absolute cell numbers were not used in analysis. Surface expression of CD45.1 served as a marker of donor derived cells and was detected using the A20 monoclonal antibody.

For adoptive transfers of bone marrow from young and aged mice into young recipient mice, bone marrow was harvested from leg bones of young and aged F1 (FVBN x CBA) mice. Cells from individual donors were not pooled. Bone marrow was washed in Hanks' Balanced Salt Solution (Life Technologies, Rockville Maryland, Cat. 14025) and suspended to 10 million cells per ml. Young recipient mice received lethal irradiation (950 rads) 20 hours prior to adoptive transfer. Recipient mice received 5 million cells from either a young or aged donor via tail vein injection. Neomycin sulfate and polymyxin B sulfate were used as indicated above. Five weeks after adoptive transfer, bone marrow was harvested from young recipient mice for analysis. Gross inspection of young and aged mice was performed prior to and after euthanasia. Mice displaying signs of tumors or other abnormalities were eliminated from analysis.

Flow cytometric analysis

Cells were flushed from the leg bones with ice-cold staining medium [RPMI 1640 deficient for biotin, flavin, and phenol red (Irvine Scientific, cat# 9826, Santa Ana, CA), with 10 mM HEPES pH 7.2, 0.02% sodium azide, 1 mM EDTA and 3% newborn calf serum] using a 22-g needle. Freshly isolated cells were washed and suspended at 3 x 10^7/ml in staining medium. Cells were treated for 10 minutes on ice with purified antibody (2.4G2) to block Fc receptors. Cells were incubated with primary antibodies for 20 minutes followed by three washes, then incubated with streptavidin-Cy5-PE for 15 minutes, followed by three washes. Cells were re-suspended in staining medium with propidium iodide (1 μg/ml) to exclude dead cells.

Primary antibodies specific for the following were used: CD24(30F1)-Alexa594, CD24-Cascade Blue, CD43(S7)-PE, 493-Biotin, CD45.1 (A20)-Cascade Blue, AA4.1-Alexa 594, AA4.1-Biotin, B220(RA3-6B2)-APC, B220-Biotin, DX5-Biotin, IgM(331)-Alexa 594, IgM-Biotin, IgM-Cascade Blue and Ly6C(AL21)-Biotin. StrepAvidin-Cy5PE was used to reveal biotinylated reagents. Antibodies were purchased from PharMingen (San Diego, CA), eBioscience (San Diego, CA) or CALTAG (Burlingame, CA). CD24, CD45.1, AA4.1 and IgM antibodies were purified and conjugated to Cascade Blue and Alexa 594 (Molecular Probes, Inc., Eugene, OR) in our laboratory using standard methods. *rag2* gene expression (NG transgenics and RAG2-GFP knock-in) was evaluated by detection of GFP using 488nm excitation (argon laser) and a band pass filter of 510 ± 20. V(D)J recombinase activity was evaluated by detection of VEX (H2-SVEX transgenic) using 407nm excitation (krypton laser) and a band pass filter of 510 ± 20. Flow cytometry was performed on a 3-laser (argon, krypton and dye laser tuned to 600 nm), 10 parameter FACSVantage from BD Biosciences Immunocytometry Systems. Post-hoc compensation and data analyses were performed using FlowJo software (Tree Star, San Carlos, CA). Stages of B cell development were identified by previously used methods (18).

Statistical analysis

Differences between groups were evaluated by either the Welch-Aspin t or the Student's t Test (depending on whether or not there was significant heterogeneity of variances as determined by Levene's Test for Equality of Variances). Analysis of Covariance was used to evaluate differences between groups while controlling for the number of pro-B cells. Differences between groups were defined as statistically significant when their probability of occurrence by chance alone was lower than 0.05. For tests of heterogeneity of variances a threshold of 0.1 was used so as to be conservative in order to protect against violations of the assumption of heterogeneity. All correlations represented are Pearson Correlations. All analyses were performed using SPSS version 10.0 for Macintosh (SPSS, Chicago IL). Statistical analyses were performed under the supervision of Mr. Steven Baker, Dept. of IS, UMMS.

RESULTS

Age-associated reduction in pre-B cells is correlated with a reduction in the percent of pro-B cells that express *rag2*.

Reduced expression of *rag* in developing pro-B cells could explain the reduced numbers of pre-B cells in aged mice. However, it remains unknown if *rag* expression is reduced specifically within pro-B cells of aged mice. In addition, the reduction in numbers of pre-B cells is highly variable between aged mice even of the same inbred strain (90, 95, 101), and it is not known if *rag* expression is reduced in all aged mice, or only in those in which the number of pre-B cells is reduced.

To address these questions we compared numbers of pro- and pre-B cells in young and aged NG transgenic mice. In these mice GFP serves as a reporter of *rag2* gene expression (24, 80). This allowed us to compare *rag2* expression in pro-B cells with the absolute numbers of pre-B cells in young versus aged mice. The NG transgene contains 180 kb of the *rag* genomic locus in which GFP is inserted in place of the major *rag2* exon. The endogenous *rag* locus is intact and retains all of the normal regulatory elements necessary for developmentally-regulated *rag* expression. These mice retain normal generation of lymphocytes. Using flow cytometric analysis of GFP, the expression of *rag2* can be accurately assessed at the single-cell level in conjunction with cell surface markers that define distinct stages of B cell development (24, 80). This method of analysis provides two advantages over previous determinations of *rag* expression in aged mice: 1) measurement of the level of *rag2* expression in single, live cells rather than in lysates from total bone marrow, and 2) a measure of *rag2* expression within cells at specific stages of B cell development.

In order to examine reduced numbers of pre-B cells and *rag2* expression in pro-B cells we analyzed bone marrow from young (2-3 month) and aged (26-27 months) NG transgenic (FVBN x CBA) F1 mice. Representative analysis of pro-B cells ($B220^{+}CD43^{+}AA4.1^{+}$) and pre-B cells ($B220^{+}CD43^{-}AA4.1^{+}CD24^{++}$) is shown in Figure

2A. Inclusion of AA4.1 in this analysis ensures that mature re-circulating B cells and non-B lineage cells are excluded and allows the accurate identification of $AA4.1^{+}CD24^{++}$ pre-B cells (17, 120-122) (and data not shown). We found that the number of pre-B cells was significantly lower in aged mice (n=5) as compared to young mice (n=6) (mean = 1.3 $\pm$0.79 versus 4.1 $\pm$ 1.0 million cells; p= 0.001; Figure 2B and Table 1A). In addition, the number of pro-B cells was marginally, but statistically significant, lower in aged mice as compared to young mice (mean = 0.70 $\pm$ 0.12 versus 1.0 $\pm$ 0.29 million cells; p= 0.035). Using an analysis of covariance we determined that the age-related reduction in number of pre-B cells is still significant when controlling for differences in the number of pro-B cells between young and aged mice. This suggests that the loss of pre-B cells in aged mice is not solely the result of a smaller precursor pool of pro-B cells.

FIGURE 2. Reduced *rag2* expression in pro-B cells is correlated with lower pre-B cell numbers in aged mice.

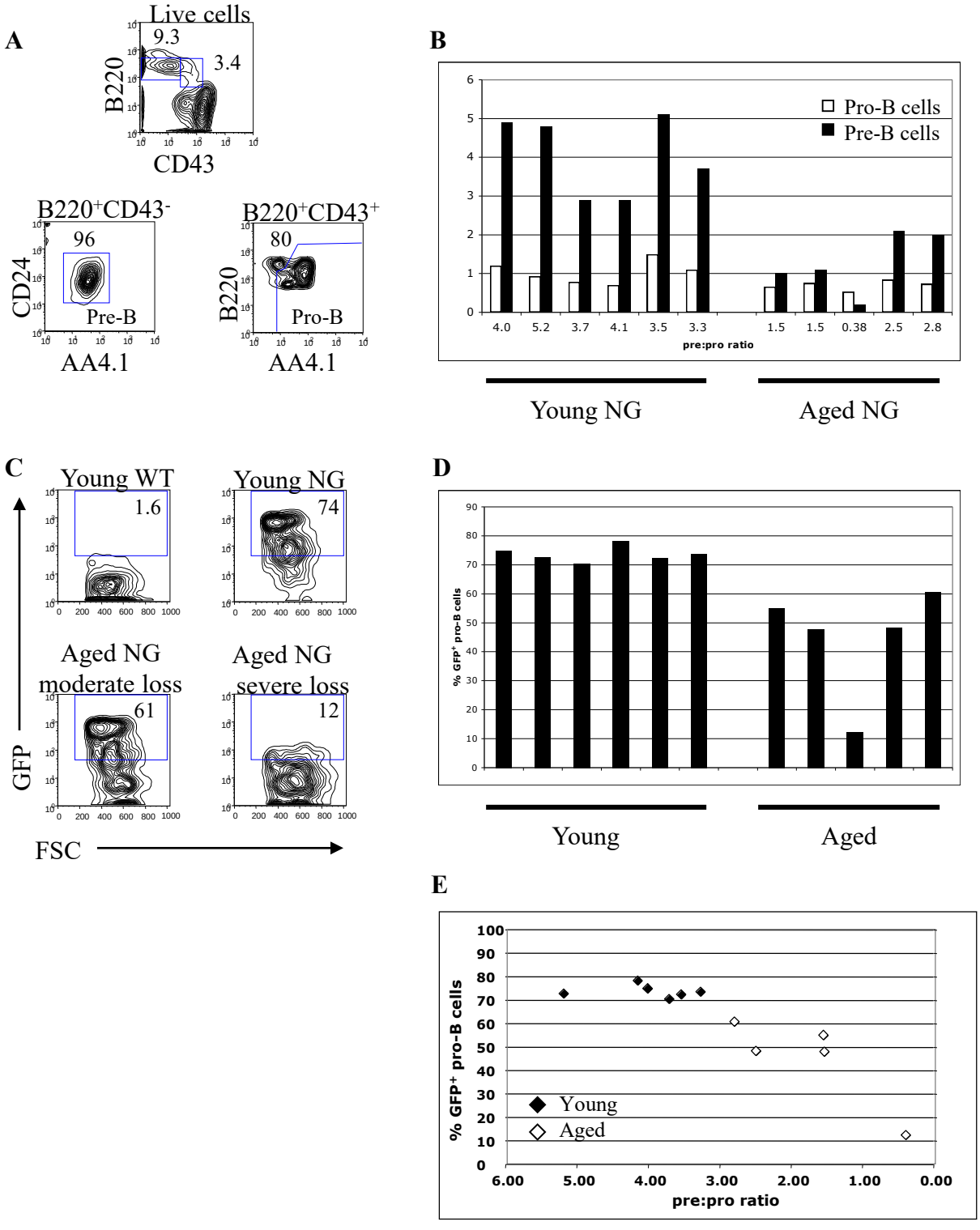


FIGURE 2. Reduced *rag2* expression in pro-B cells is consistent with lower numbers of pre-B cells in aged mice. Bone marrow from young (2-3.5 months) and aged (26-27 months) NG mice, and wild type F1 (FVBN x CBA) controls was stained with antibodies to B220, CD43, AA4.1 and CD24 and analyzed by flow cytometry. (A) Flow cytometric analysis from a representative young wild type mouse. Labels above panels indicate the phenotype of the cells displayed in each graph, and the number reflects the percent of displayed cells within the gate. The pre:pro ratio is calculated by dividing the number of pre-B cells ($B220^{int}CD43^{-}AA4.1^{+}CD24^{++}$) by the number of pro-B cells ($B220^{+}CD43^{+}AA4.1^{+}$). (B) Numbers of pro- and pre-B cells in bone marrow of young and aged mice. Open bars represent pro-B cells, closed bars represent pre-B cells. (C) Flow cytometric analysis of GFP (a reporter of *rag2* expression in NG mice) in pro-B cells from young and aged NG mice. Bone marrow pro-B cells from wild type, young NG, aged NG with a moderate loss of GFP expression and an aged NG with a severe loss of GFP expression are displayed. The numbers within the gates depict the percent of pro-B cells that express GFP. (D) The percent of pro-B cells that are GFP^{+} are shown for the same mice depicted in B. (E) The percent of pro-B cells that are GFP^{+} and the pre:pro ratio are displayed for each NG mouse shown in B and D. Closed diamonds represent young mice, open diamonds represent aged mice.

TABLE 1. Statistical Analysis of Data Obtained from Young and Aged NG Mice. Analysis of the young and aged NG mice shown in Figure 2. (A) The mean, standard deviation and significance of differences in means between young and aged mice groups are shown. (B) The correlations between traits are shown.

A. Significance of Differences Between Young and Aged Groups

	young	aged	p-value
Number of pro-B cells (millions)	1.0 ± 0.29	0.70 ± 0.12	0.035
Number of pre-B cells (millions)	4.1 ± 1.0	1.3 ± 0.79	0.001
Pre:pro ratio	4.0 ± 0.67	1.7 ± 0.94	0.001
Percent of pro-B cells that are $CD24^{high}$	61 ± 4.1	45 ± 10	0.005
Percent of pro-B cells that are GFP^{+}	74 ± 2.7	45 ± 19	0.026

B. Correlation Between Characteristics

	n	r	p-value
Percent of pro-B cells that are that GFP^{+} and number of pre-B cells	11	0.80	0.003
Percent of pro-B cells that are GFP^{+} and pre:pro ratio	11	0.88	<0.001

(n = number of mice, r = correlation coefficient, p = significance)

In addition to the number of pre-B cells, the ratio of pre:pro B cells can provide an indicator of pre-B cell dynamics. Reduced pre-B cell numbers could be due to reduced generation, and or maintenance of pre-B cells rather than solely the result of reduced numbers of precursors in the pro-B cell stage. The pre:pro ratio was significantly lower in aged versus young mice (mean = 1.7 $\pm$0.94 versus 4.0 $\pm$0.67; p= 0.001; Figure 2A), suggesting that the reduction in the numbers of pro-B cells in aged mice does not completely account for the reduction in the numbers of pre-B cells. Expression of CD24 increases as cell mature within the pro-B cell stage (123). Using CD24 as an indicator of maturation within the pro-B cell stage (18, 123), we observed that the percent of pro-B cells that are $CD24^{high}$ is significantly lower in aged mice as compared to young mice (mean = 45 $\pm$10 versus 61 $\pm$ 4.1 %; p= 0.005; Table 1A). Together, these observations support the concept that B cell development is altered in aged mice as early as the pro-B cell stage and that reduced developmental progression of pro-B cells may contribute to the lower numbers of pre-B cells observed in aged mice.

Rag2 expression is essential to V(D)J recombination and generation of an intact IgH chain which is required for pre-B cell production. Expression of *rag2* is reduced in total bone marrow of aged mice, but it is unknown if expression is lower within pro-B cells. Therefore, we compared *rag2* expression in the bone marrow of young and aged mice to determine if *rag2* expression is lower within pro-B cells, and if this reduction is correlated with the reduction in the number of pre-B cells. Flow cytometric analysis of the young and aged NG transgenic mice was used to determine the percent of pro-B cells that express GFP as a reporter of *rag2* expression (Figure 2C and Figure 2D). The percent of pro-B cells that are GFP^{+} is significantly lower in aged mice as compared to young mice (mean = 45 $\pm$19 versus 74 $\pm$ 2.7 percent; p= 0.026; Table 1A). This indicates that the percent of pro-B cell that express *rag2* is reduced in aged mice. In the pro-B cells that do express GFP, the per cell levels, as measured by the mean fluorescent intensity of GFP, was similar between young mice and aged mice. This suggests that the levels of

rag2 per pro-B cell are similar in young and aged mice, even though fewer pro-B cells express *rag2* in aged mice.

The loss of *rag2* expression in pro-B cells is correlated with reduced numbers of pre-B cells. We found that the percent of pro-B cells that are GFP^+ is correlated with both the number of pre-B cells ($n=11$, $r=0.80$, $p=0.003$) and the pre:pro ratio ($r= 0.88$, $p<0.001$) (Table 1B and Figure 2E). This relationship is also evident in Figure 2B, as the aged mouse with the lowest percent of GFP^+ pro-B cells is the same mouse that has the lowest number of pre-B cells and pre:pro ratio.

Together, these results indicate that the percent of pro-B cells that express *rag2* is reduced in aged mice and is correlated with the reduction in pre-B cell numbers. This supports the hypothesis that reduced numbers of pre-B cells in aged mice are due to defects in B cell development that occur during the pro-B cell phase of development and that affect expression of *rag2*.

Of the five aged mice, there is one mouse in which the number of pre-B cells, the pre:pro ratio and the percent of pro-B cells that are GFP+ are considerably lower than the other 4 mice. This raises the concern that one aged mouse could account for the majority of the differences observed between young and aged mice. A statistical analysis of the young and aged mice was conducted in which this mouse was excluded. Among the remaining four aged mice, the mean number of pre-B cells was 1.6 ± 0.57 million and the difference between young and aged mice was still significant ($p= 0.001$). In addition, the mean pre:pro ratio was 2.1 ± 0.65 and difference between young and aged mice was still significant ($p=0.002$). Furthermore, the mean percent of pro-B cells that are GFP^+ in the four aged mice was 53 ± 6.1 and the difference between young and age mice was still significant ($p= 0.004$). Thus exclusion of the aged mouse with the most extreme phenotype does not alter the observations that the number of pre-B cells, the pre:pro ratio

and the percent of pro-B cells that are GFP^+ are all significantly reduced in aged, as compared to young, mice.

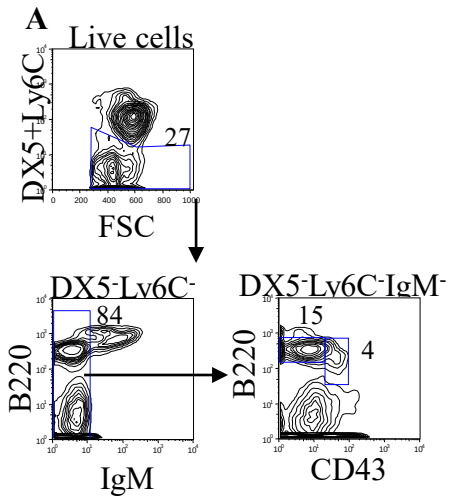
Reduced numbers of pre-B cells in aged mice is correlated with reduced RAG2 protein levels in pro-B cells.

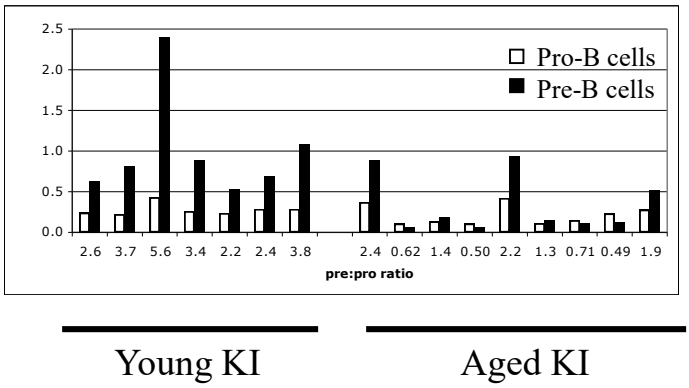
We next analyzed B cell development in RAG2-GFP knock-in (KI) mice (81). These KI mice are accurate reporters of RAG2 levels in single cells, and have two important advantages compared to NG mice: 1) the GFP reporter is located within the endogenous *rag2* locus, and 2) GFP is expressed as a fusion protein with RAG2 and thus serves as a direct reporter of cellular RAG2 protein levels. In these mice the RAG2-GFP fusion protein maintains normal RAG enzymatic activity and B cell development is not altered. GFP levels per cell and the percent of pro-B cells that are GFP^+ are lower in these mice compared to the NG transgenic mice. This difference could be due to the single insertion of GFP in the KI compared to a multi-copy NG transgene as well as differences in protein half-life for the RAG2-GFP fusion in the KI compared to GFP in the transgenic. We analyzed RAG2-GFP expression in pro-B cells harvested from young (4-7 months) and aged (23-28 months) RAG2-GFP KI mice. Representative flow cytometric analysis of pro-B cells ($IgM^-B220^+CD43^+$) and pre-B cells ($IgM^-B220^+CD43^-$) is shown in Figure 3A. Cells that could contaminate pro-B and pre-B cell populations were excluded based on expression of either Ly6C (myeloid, activated T and B cells, plasma cells, plasmacytoid dendritic cells) or DX5 (NK, NKT) (124, 125). This is functionally equivalent to using AA4.1 to positively identify cells of pro-B and pre-B cells (data not shown).

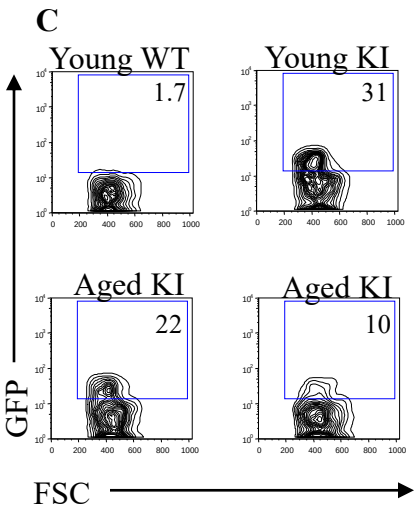
As with the NG mice, the percent of pro-B cells that express *rag2* was significantly lower in the aged KI mice as compared to the young KI mice (mean = 14 $\pm$ 12 versus 33$\pm$ 3.6 %; p= 0.001; Figure 3D and Table 2A). Both models demonstrate that *rag2* expression in pro-B cells is lower in aged mice. Also, because the KI reporter is a RAG2-

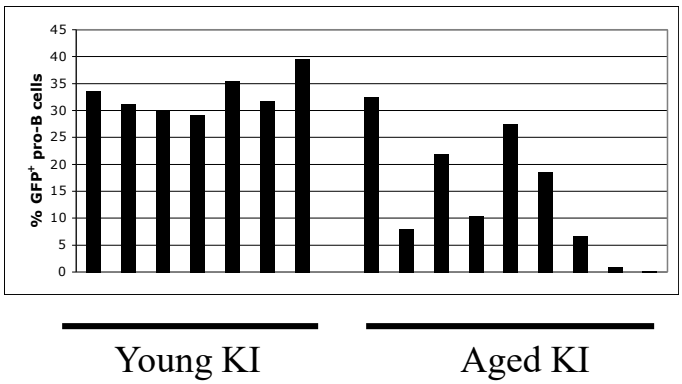
GFP fusion protein, the data suggest that protein levels of RAG2 are lower in the pro-B cells of aged mice.

FIGURE 3. Reduced RAG2 protein levels in pro-B cells are correlated with lower pre-B cell numbers in aged mice.









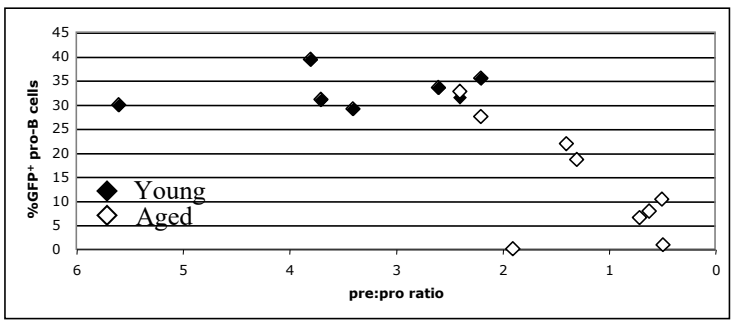


FIGURE 3. Reduced RAG2 protein levels in pro-B cells are consistent with lower numbers of pre-B cells in aged RAG2-GFP knock-in mice. Bone marrow from young (4-7 months) and aged (23-28 months) RAG2-GFP knock-in mice was stained with antibodies to Ly6C, DX5, IgM, B220 and CD43 and analyzed by flow cytometry. (A) Representative flow cytometric analysis of a young, wild type mouse. Label above panels indicate the phenotype of cells displayed in each graph, and the number near each gate reflects the percent of displayed cells within the gate. Pro-B cells ($Ly6C^-DX5^-IgM^-B220^+CD43^+$) and pre-B cells ($Ly6C^-DX5^-IgM^-B220^+CD43^-$) are shown in the right panel. (B) Numbers of pro- and pre-B cells in bone marrow of young and aged mice. Open bars represent pro-B cells, closed bars represent pre-B cells. The pre:pro ratio was calculated by dividing the number of pre-B cells by the number of pro-B cells. (C) Analysis of GFP in pro-B cells of RAG2-GFP knock-in mice. Bone marrow from young wild type, young RAG2-GFP knock-in, aged RAG2-GFP knock-in with a moderate loss of GFP expression, and an aged RAG2-GFP knock-in with severe loss of GFP expression are displayed. The numbers within the gates depict the percent of pro-B cells that express GFP. (D) The percent of pro-B cells that are GFP+ are displayed for the same mice shown in B. (E) The percent of pro-B cells that are GFP+ and the ratio of pre-B to pro-B cells are displayed for each knock-in mouse shown in B and D. Closed squares represent young mice, open squares represent aged mice.

TABLE 2. Statistical Analysis of Data Obtained from Young and Aged RAG2-GFP Knock-in Mice.

Analysis of the young and aged RAG2-GFP knock-in mice shown in Figure 3. (A) The mean, standard deviation and significance of differences in means between young and aged mice groups are shown. (B) The correlations between traits are shown.

A. Significance of Differences Between Young and Aged Groups

	young	aged	p-value
Number of pro-B cells (millions)	0.28 ± 0.07	0.21 ± 0.12	NS
Number of pre-B cells (millions)	1.0 ± 0.64	0.33 ± 0.35	0.019
Pre:pro ratio	3.4 ± 1.2	1.3 ± 0.75	0.001
Percent of pro-B cells that are GFP^+	33 ± 3.6	14 ± 12	0.001

B. Correlation Between Characteristics

	n	r	p-value
Percent of pro-B cells that are $GFP^+$and number of pre-B cells	16	0.575	0.020
Percent of pro-B cells that are $GFP^+$and pre:pro ratio	16	0.709	0.002

(n = number of mice, r = correlation coefficient, p = significance, NS = not significant)

The number of pre-B cells was significantly lower in aged KI mice as compared to young KI mice (mean = 0.33 $\pm$0.35 versus 1.0 $\pm$ 0.64 million cells; p= 0.019;Table 2A). We found that the number of pro-B cells was not significantly different between young and aged mice (mean =0.28 $\pm$0.070 and 0.21 $\pm$ 0.12 million cells). In addition, the pre:pro ratio was lower in the aged mice as compared to young mice (mean = 1.3 $\pm$0.75versus 3.4 $\pm$1.2; p= 0.001; Table 2A). Similar to our data with the NG mice, these results suggest that the reduction in the numbers of pre-B cells in aged mice is not solely due to a reduction in the size of the precursor population of pro-B cells, and may also reflect reduced development of pro-B cells and generation of pre-B cells.

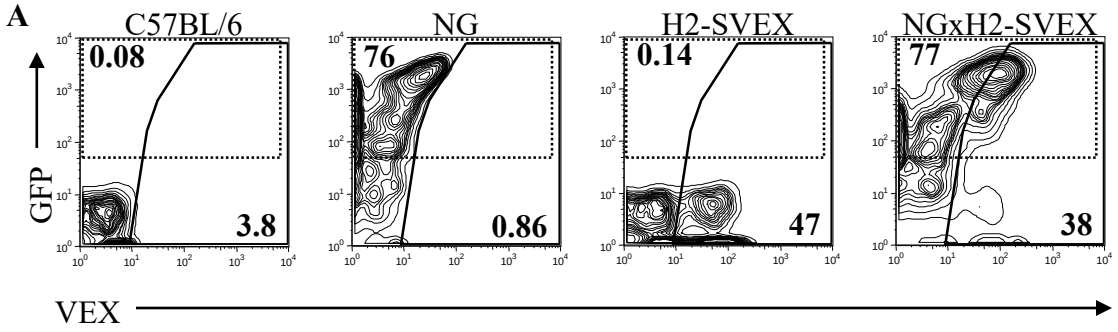
Furthermore, we observed that the percent of pro-B cells that express GFP was correlated with both the number of pre-B cells (n=16, r=0.575, p=0.020) and the pre:pro ratio (n= 16, r=0.709, p=0.002) (Table 2B). The relationship is also evident in the data depicted in Figure 3E: the aged RAG2-GFP KI mice that displayed the lowest numbers of pre-B cells and pre:pro ratios were the same mice that exhibited the lowest percent of pro-B cells expressing RAG2-GFP. Taken together, our analyses of both the NG transgenic and RAG2-GFP knock-in mice indicate that the reduction in numbers of pre-B cells is correlated with reduced *rag2* expression in pro-B cells.

rag2 expression, V(D)J recombinase activity, and the pre:pro ratio are reduced in aged mice.

V(D)J recombinase activity can be demonstrated in live cells using H2-SVEX transgenic mice which carry a fluorescent V(D)J recombination reporter substrate (82). Cells that undergo V(D)J recombination of the transgene express VEX , a spectrally-distinct variant of GFP (104, 126), and are easily detected by flow cytometry. In addition, the percent of VEX^+ cells within a population reflects the level of *rag* expression (82). We crossed NG transgenic mice to H2-SVEX mice to generate double

transgenic mice (C57BL/6 background) in which *rag2* expression could be measured in conjunction with V(D)J recombinase activity. Figure 4A displays expression of GFP and VEX in live pro-B cells from wild type (C57BL/6), NG transgenic, H2-SVEX transgenic and NG x H2-SVEX double transgenic mice. Cells expressing either fluorescent reporter are clearly resolved from each other and from non-expressing cells. GFP is clearly detected in NG and double transgenic mice, but not in wild type or H2-SVEX mice. Also, VEX is clearly detected in H2-SVEX and double transgenic mice, but not in wild type or NG mice.

FIGURE 4. *Rag2* expression, V(D)J recombinase activity and the pre:pro ratio are reduced in aged mice.



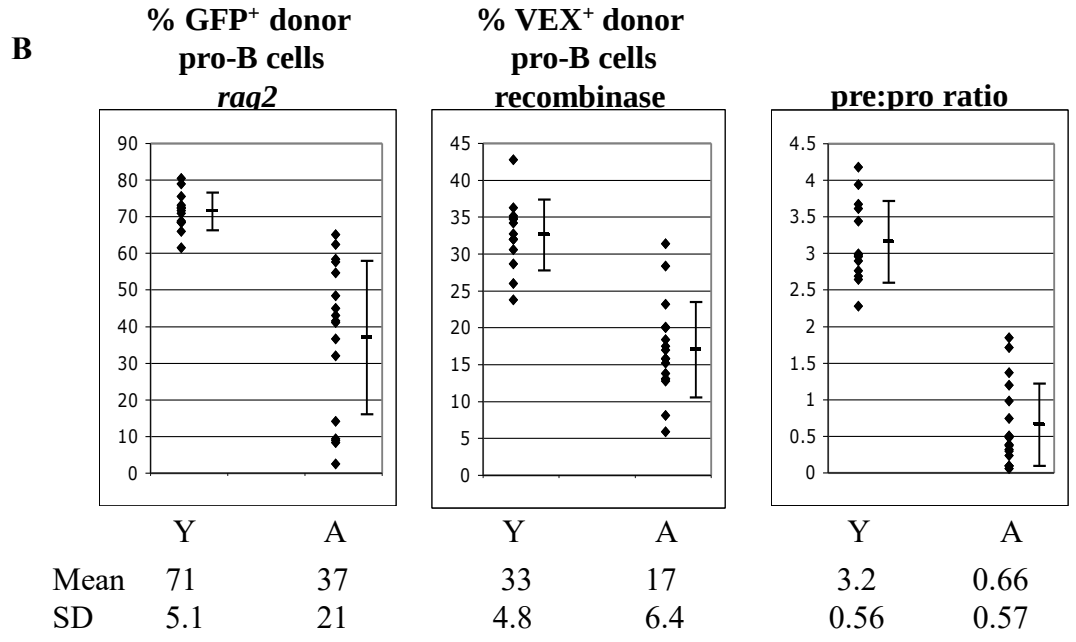


FIGURE 4. *Rag2* expression, V(D)J recombinase activity and the pre:pro ratio are reduced in aged mice. (A) Simultaneous flow cytometric analysis of reporters for both RAG2 expression and recombinase activity. NG x H2-SVEX double transgenic mice were generated in which GFP serves as a reporter of RAG2 expression and VEX serves as a reporter of V(D)J recombinase activity. Bone marrow from wild type, NG transgenic, H2-SVEX transgenic and NG x H2-SVEX double transgenic mice were stained with antibodies to IgM, B220 and CD43. GFP and VEX expression in pro-B cells ($IgM^-B220^+CD43^+$) are displayed. The numbers within each gate reflect the percent of displayed cells within the gate. The dotted line depicts the gate used to define GFP expression, and the solid line depicts the gate used to define VEX expression. The VEX gate is drawn based on the expression of VEX in the H2-SVEX transgenic compared to the NG transgenic; the gate is non-rectangular so as to exclude the bright "GFP-only" cells that cannot otherwise be accurately excluded in the analysis of VEX in the double transgenic mice due to the limitations of flow cytometric compensation correction. (B) Bone marrow was harvested from wild type, CD45.2 young (three to four months at time of harvest) and aged (23-29 months at time of harvest) recipient mice five to six weeks after adoptive transfer of bone marrow from NG x H2-SVEX double transgenic CD45.1 mice. Pro-B ($IgM^-B220^+CD43^+$), and pre-B cells ($IgM^-B220^+CD43^-$) of donor origin were identified based on expression of CD45.1. The left and middle panels display the mean, standard deviation and distribution of the percent of donor origin pro-B cells that are GFP^+ and VEX^+ respectively. The right panel displays the mean, standard deviation and distribution of the pre:pro ratio. The pre:pro ratio was calculated by dividing the percent of bone marrow that was pre-B cells by the percent that of bone marrow that was pro-B cells for each recipient mouse. Y denotes young mice, A denotes aged mice.

Because *rag2* is essential for V(D)J recombination, the reduction in *rag2* expression in aged mice may result in a corresponding decrease in V(D)J recombinase activity. Alternatively, it was possible that the reduction in *rag2* expression is not of sufficient magnitude to affect V(D)J recombinase activity and that other age-related defects actually restrict B cell development. We addressed this by using NG x H2-SVEX double transgenic mice and adoptive transfers into young and aged mice. This provided two advantages: 1) we were able to isolate effects of the aged bone marrow microenvironment independent of cell-intrinsic defects and 2) this was an expedient alternative to aging double transgenic mice. NG mice were back-crossed on to the C57BL/6 background for six generations were mated to H2-SVEX mice. Whole bone marrow from young NG x H2-SVEX CD45.1 double transgenic mice was transferred into young (3 months) and aged (28 months) C57BL/6 CD45.2 lethally-irradiated recipient mice. Five to six weeks after transfer we compared *rag2* expression and V(D)J recombinase activity in donor-derived pro-B cells and the pre:pro ratio of donor-derived cells. Pro- and pre-B cells derived from transferred hematopoietic stem cells or very early progenitors present in the transferred marrow were identified based on expression of CD45.1.

The percent of donor-derived pro-B cells that express *rag2* was significantly lower in aged recipients as compared to young recipients (mean = 37 $\pm$21 versus 71$\pm$5.1; $p<0.001$; Figure 4B and Table 3A). This reduction is comparable to the reduction we observed in the NG transgenic (Figure 2 and Table 1) and RAG2-GFP knock-in mice (Figure 3 and Table 2). In addition, V(D)J recombinase activity, as indicated by the percent of pro-B cells that are VEX^+, was also significantly lower in pro-B cells from aged recipients as compared to young recipients (mean = 17$\pm$6.4 versus 33$\pm$4.8; $p<0.001$; Figure 4B and Table 3A). This is the first analysis of V(D)J recombinase activity in pro-B cells in aged mice and it suggests that the reduction in *rag2* expression in pro-B cells of aged mice is sufficient to result in reduced V(D)J recombinase activity.

TABLE 3. Statistical Analysis of Data Obtained from Young and Aged C57BL/6 Recipient Mice Following Adoptive Transfer of NG x H2-SVEX Transgenic Bone Marrow.

A. Significance of Differences Between Young and Aged Groups

	young	aged	p-value
% of donor pro-B cells that are GFP^+	71 $\pm$ 5.1	37 $\pm$ 21	<0.001
% of donor pro-B cells that are VEX^+	33 $\pm$ 4.8	17 $\pm$ 6.4	<0.001
Pre:pro ratio	3.2 $\pm$ 0.56	0.66 $\pm$ 0.56	NA
Log of pre:pro ratio	1.1 $\pm$ 0.18	-0.83 $\pm$ 1.0	<0.001

B. Correlation Between Characteristics, All Mice (n = 30)

	LN pre:pro ratio	% of pro-B cells that are GFP+	% of pro-B cells that are VEX+
Log pre:pro ratio		r=0.812 p<0.001	r=0.860 p<0.001
% of pro-B cells that are GFP^+	r=0.812 p<0.001		r=0.835 p<0.001
% of pro-B cells that are VEX^+	r=0.860 p<0.001	r=0.835 p<0.001	

C. Correlation Between Characteristics, Aged Mice (n = 17)

	LN pre:pro ratio	% of pro-B cells that are GFP+	% of pro-B cells that are VEX+
Log Pre:pro ratio		r=0.550 p=0.022	r=0.670 p=0.003
% of pro-B cells that are GFP^+	r=0.550 p=0.022		r=0.634 p=0.006
% of pro-B cells that are VEX^+	r=0.670 p=0.003	r=0.634 p=0.006	

(n = number of mice, r = correlation coefficient, p = significance, NA = not applicable as distribution is not normal).

TABLE 3. Statistical Analysis of Data Obtained from Young and Aged C57BL/6 Recipient Mice Following Adoptive Transfer of NG x H2-SVEX Transgenic Bone Marrow.

Analysis of the young and aged C57BL/6 recipient mice from Figures 3 and 4. (A) The mean, standard deviation and significance of differences in means between young and aged mice groups are shown. (B) The correlations between traits within all mice (included young and aged) are shown. (C) The correlations between traits within the aged mice are shown.

In addition, we analyzed the pre:pro ratio of $CD45.1^+$ donor-derived cells as a measure of pre-B cell numbers in the young and aged adoptive transfer recipients. We used the pre:pro ratio because absolute numbers of donor derived cells were not comparable between mice due to variation in degree of engraftment in individual mice. The donor pre:pro ratio was lower in the aged recipient mice as compared to the ratio in young recipient mice (mean = 0.66 $\pm$ 0.56 versus 3.2 $\pm$ 0.56). Because the ratios did not have a normal distribution, we conducted a student's t-test on the log (LN) ratios, which were normally distributed. The mean log ratio was significantly lower in aged recipient mice than in young recipient mice (mean = -0.83 $\pm$ 1.0 versus 1.1$\pm$0.18; $p<0.001$; Table 3A).

The age-associated reduction in *rag2* expression, V(D)J recombinase activity in pre:pro ratio might be the result of independent age-associated defects or these could be interrelated. To address this, we conducted statistical analyses to determine if these traits were correlated in the population of mice as a whole (n=30), and if these correlations were also observed if the analysis was restricted to the aged recipient mice (n=17). As reported in Table 3B and C, all three parameters (the percent of pro-B cells that were GFP^+, the percent of pro-B cells that were VEX^+ and the pre:pro ratio) were correlated in both the entire population of mice (n=30) and in the aged group (n=17). These correlations are also apparent when individual aged mice are examined for each parameter: mice with extensive reduction in *rag2* expression in pro-B cells also had an extensive loss of V(D)J recombinase activity in pro-B cells, and significant reduction of the pre:pro ratio (data not shown). In contrast, mice that had a minor reduction in *rag2* expression also had only minor reductions in V(D)J recombinase activity and the pre-pro ratio.

The bone marrow microenvironment controls *rag2* expression and V(D)J recombinase activity in pro-B cells and the pre:pro ratio.

The use of an adoptive transfer model also enabled us to determine if the age-associated defects in B cell development are due to changes in the hematopoietic microenvironment of the bone marrow or due to changes that are cell–intrinsic. We observed that donor-derived pro-B cells displayed lower *rag2* expression and V(D)J recombinase activity and there was a lower pre:pro ratio following bone marrow transfer into aged as compared to young recipient mice (Figure 4). This was observed for transferred cells from young donor mice, thus indicating that age-associated alterations specific to the bone marrow microenvironment are sufficient to produce these defects in B cell development.

Young bone marrow microenvironment restores *rag2* expression and the pre:pro ratio.

The presence of defects in B cell development due to changes in the bone marrow hematopoietic microenvironment does not preclude the existence of age-related, cell-intrinsic defects in B cell development. To determine if cell-intrinsic defects affect *rag2* expression in pro-B cells, and the pre:pro ratio, we conducted adoptive transfer experiments in which bone marrow from aged and young donor mice was transferred into young recipient mice. The NG mice shown in Figure 2 (four of the six young and all of the aged) were used as sources of bone marrow for adoptive transfer into lethally irradiated young, wild type recipient mice. Donor bone marrow was not pooled in this experiment; each recipient mouse received an adoptive transfer from either a young or aged donor. Using this experimental design, progenitors from young and aged mice were evaluated after differentiation within the same microenvironment; the bone marrow of a young recipient.

Prior to adoptive transfer all five of the aged donor mice displayed significantly) lower pre:pro ratios than those of the young donor mice (mean = 1.7 ± 0.94 versus

3.7±0.41; p=0.006; Figure 5A and Table 4A). The percent of pro-B cells that express *rag2* was also significantly lower in the five aged donor mice than in the young donor mice (mean = 45±19% versus 75±2.5%; p= 0.023; Figure 5B and Table 4A).

FIGURE 5. Pro-B cells derived from young and aged sources display similar *rag2* expression and pre:pro ratios in young hosts.

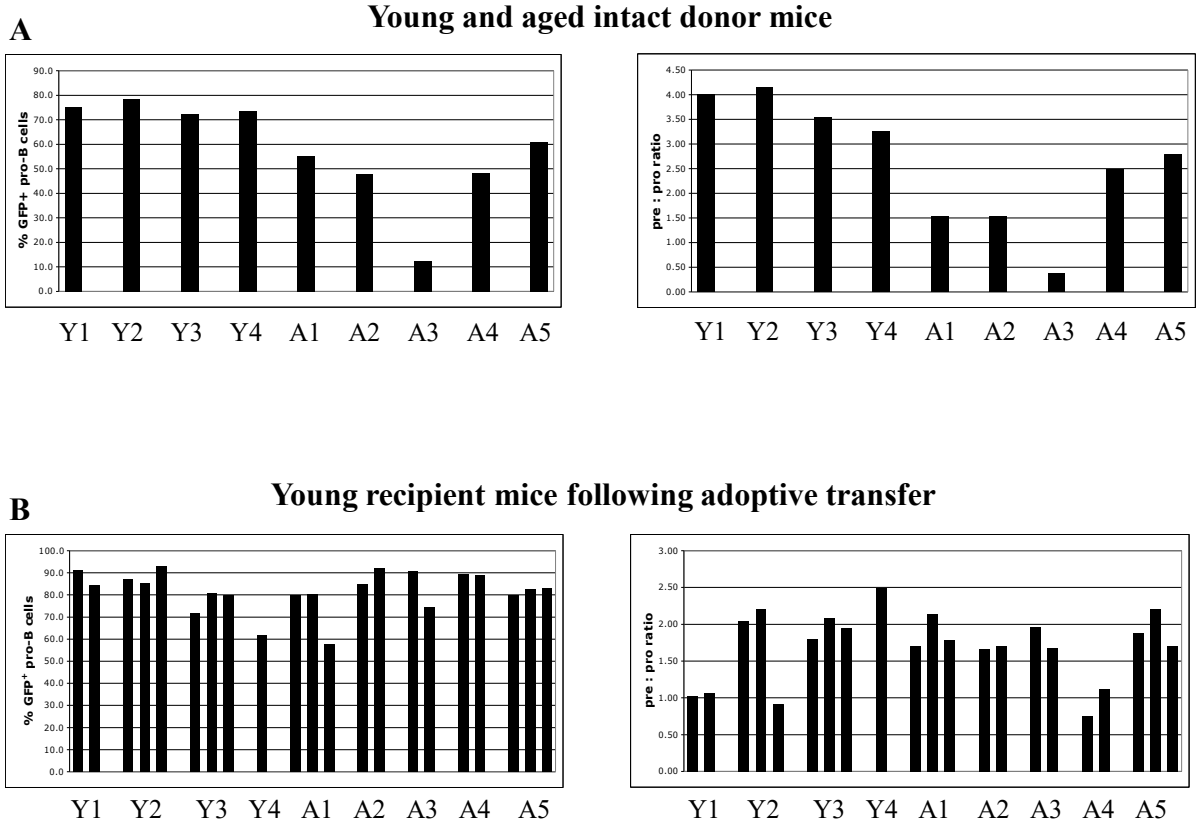


FIGURE 5. Pro-B cells derived from young and aged sources display similar *rag2* expression and pre:pro ratios in young hosts. Bone marrow from young (3 months) and aged (26-27 months) NG mice were transferred into lethally irradiated young (2 months at time of transfer) recipient mice. (A) GFP expression in pro-B cells (left panel) and pre:pro ratio (right panel) in young and aged donor mice at time of transfer. These mice are four of the six young, and all of the aged mice shown in Figure 2. To aid comparisons, each young mouse is given a designation, Y1-Y4, and each aged mouse A1-A5, depicted between the two panels. (B) Bone marrow harvested from young recipient mice five weeks after transfer of bone marrow from either a young or aged donor. Pro-B cells were defined as $IgM^{-}B220^{+}CD43^{+}AA4.1^{+}$, pre-B cells were defined as $IgM^{-}B220^{+}CD43^{-}AA4.1^{+}CD24^{++}$. The percent of pro-B cells that are GFP^{+} and the pre:pro ratios are shown. The pre:pro ratio of two young intact NG mice used as controls in this experiment were 1.2 and 1.3. The designations Y1-Y4 and A1-A5 reflect the source of the transferred bone marrow as shown in A.

TABLE 4. Statistical Analysis of Data Obtained from Cells Harvested from Donor Mice and Recipient Mice Following Adoptive Transfer of NG Transgenic Bone Marrow.

Analysis of the young and aged donor mice from Figure 4A are displayed. (A) The mean, standard deviation and significance of differences in means between young donors and aged donor mice are shown. Data is from four of the six young mice displayed in Figure 2 (the mice that served as donor mice for adoptive transfer). (B) Analysis of the young adoptive transfer recipient mice from Figure 4B are shown.

A. Comparisons of Bone Marrow from Young and Aged Donor Mice

	young	aged	p-value
Pre:pro ratio	3.7 $\pm$ 0.41	1.7 $\pm$ 0.94	0.006
Percent of pro-B that are GFP^+	75 $\pm$ 2.5	45 $\pm$ 19	0.023

B. Comparisons of Bone Marrow from Young Recipient Mice Following Adoptive Transfer of NG Transgenic Bone Marrow from Young or Aged Donors

	from young donor	from aged donor	p-value
Pre:pro ratio	1.7 $\pm$ 0.58	1.7 $\pm$ 0.40	NS
Percent of pro-B that are GFP^+	82 $\pm$ 9.8	82 $\pm$ 9.3	NS

(n = number of mice, r = correlation coefficient, p = significance, NS = not significant)

Five weeks after adoptive transfer, B cell development and *rag2* expression in young recipient mice was assessed by flow cytometry. Pro-B cell were defined as $IgM^-B220^+CD43^+AA4.1^+$ and pre-B cells were defined as $IgM^-B220^+CD43^-AA4.1^+CD24^+$. In this experiment a marker of donor cells was not available. However, efficient engraftment must have occurred because 1) recipients survived lethal irradiation, 2) in comparable experiments, engraftment in young mice routinely resulted in over 90% pro-B cells of donor origin, and 3) the expression of GFP in pro-B cells (which clearly identifies cells of NG transgenic donor origin) was high (60% - 90% of pro-B cells) in bone marrow harvested from recipient mice (Figure 5B).

We found that *rag2* expression in young recipient mice was not significantly affected by the age of the bone marrow donors (Table 4B). The percent of pro-B cells that were GFP^+ was $82\pm9.8\%$ in mice that received bone marrow from young donors and $82\pm9.3\%$ in mice that received bone marrow from aged donors (Figure 5B and Table 4B). The severity of diminished *rag2* expression in pro-B cells of aged donors was not reflected in recipient mice. Of the donors, mouse A3 (Aged #3) displayed the lowest percent of pro-B cells with *rag2* expression, less than 15% (Figure 5A). In the two young mice that received a bone marrow transfer from A3, *rag2* was expressed in greater than 70% of pro-B cells and was comparable to that of other recipient, young mice (Figure 5B). These observations indicate that the reduction in *rag2* expression in pro-B cells of aged mice is not the result of defects that are intrinsic to the developing cells.

The pre:pro ratios were also compared in young reconstituted mice and were not significantly different due to the age of bone marrow donors (Table 4B). The mean pre:pro ratio was 1.7 ± 0.40 in mice that received bone marrow from aged donors, and 1.7 ± 0.58 in mice that received bone marrow from young donors (Figure 5B and Table 4B.) Again, the severity of the age-related defect was not reflected following adoptive transfer into young hosts. Aged donor mice A1, A2 and A3 displayed the lowest pre:pro

ratios (Figure 5A), yet the seven young recipients of bone marrow from these mice displayed pre:pro ratios that were as high as those observed in mice that received bone marrow from other aged, or young, donors (Figure 5B). This indicates that the age-related reduction in the pre:pro ratio is not the result of cell-intrinsic defects in hematopoietic precursors.

These experiments indicate that *rag2* expression and V(D)J recombinase activity in pro-B cells and the number of pre-B cells are reduced in aged mice. The degree to which expression of *rag2* is reduced is correlated with the severity of loss of V(D)J recombinase activity and pre-B cell numbers. This indicates that these three defects could be the result of the same underlying cause or that loss of *rag2* expression leads to reduced V(D)J recombinase activity and in turn, fewer pre-B cells. These experiments also show that the age-associated changes in the bone marrow microenvironment are sufficient to result in loss of rag2 expression and V(D)J recombinase activity in pro-B cells and numbers of pre-B cells.

DISCUSSION

B cell development is impaired in aged mice and characterized by fewer pre-B cells and reduced expression of *rag1* and *rag2* in total bone marrow (90, 97, 98, 101, 113). Prior to this work it was unknown if expression of *rag1* or *rag2* is lower in pro-B cells. If was also unknown if age-associated defects in B cell development are due to cell-intrinsic alterations or changes in the hematopoietic microenvironment. We have found *rag2* gene expression is reduced in pro-B cells of aged mice. The reduction in *rag2* expression is consistent with reduced V(D)J recombinase activity and reduced numbers of pre-B cells, suggesting that the age-related reduction in pre-B cell numbers could be the result of diminished *rag2* expression. We also show that these defects are due to age-related defects in the bone marrow hematopoietic microenvironment as opposed to cell-intrinsic defects.

The percent of pro-B cells that express *rag2* and display V(D)J recombinase activity as measured by the transgenic reporters are reduced in aged mice. We analyzed B cell development in young and aged NG transgenic mice (24, 80) and RAG2-GFP knock-in mice (81). Robust GFP expression in the former system enables characterization of *rag2* expression in small subsets of lymphoid progenitors while the latter system enables real-time analysis of RAG2 protein. In both systems we extended the analysis of *rag2* expression in aged mice to show that expression is reduced within pro-B cells of aged mice (Figures 2, 3 and 4 and Tables 1, 2 and 3). An assessment of *rag* expression at the single cell level in pro-B cells has not been done prior to this. A strength of this approach is that we were able to determine that reduced *rag2* expression in pro-B cells of aged mice is due to a lower percent of pro-B cells that express *rag2* as opposed to a lower per cell level of expression within all pro-B cells.

It is unlikely that the reduction in GFP in aged transgenic mice is due to specific silencing of the transgene. Our results from the NG transgenic mice are consistent with those of the RAG2-GFP knock-in fusion protein mice that is unlikely to be silenced by the same mechanisms that silence repetitive transgenic DNA sequences. In addition, pro-

B cells generated from precursors from aged NG mice expressed GFP levels similar to that of pro-B cells derived from young NG mice following adoptive transfer into young host. This restoration of expression indicates that the transgene was not silenced. These observations indicate that the reduction in GFP expression in pro-B cells of aged mice reflect a loss of *rag2* expression rather than silencing of the reporter transgene.

We also used H2-SVEX mice to study V(D)J recombinase activity at the single-cell level for the first time in aged mice (82). Using NG x H2-SVEX double transgenic mice as donors, we were able to show that V(D)J recombinase activity is reduced in pro-B cells of aged mice. In H2-SVEX transgenic mice, the reporter recombination substrate provides a measure of recombinase activity independent of developmentally-regulated accessibility of the immunoglobulin loci. Thus, the reduction in recombination that we observed in aged mice is attributable to reduced expression, or activity, of the enzyme complex required for V(D)J recombinase activity. This indicates that the degree to which *rag2* expression is reduced in pro-B cells of aged mice is of sufficient magnitude to result in a decrease of V(D)J recombinase activity. This supports the hypothesis that reduced *rag2* expression results in reduced V(D)J recombinase activity, generation of the pre-BCR and transition of cells into the pre-B cell stage of development.

In addition, we found that the loss of *rag2* expression and V(D)J recombinase activity in pro-B cells was associated with the reduction in pre-B cell numbers. Importantly, aged mice exhibit these traits in association with each other, as shown by the correlations between these traits when statistical analysis considered only the aged mice (Table 3C). Aged mice with a moderate decline in *rag2* expression also displayed a moderate reduction in recombinase activity and pre:pro ratio while in other mice all three defects were more pronounced (Figure 4C). This indicates that the reduction of transcription of *rag2*, whose function is required for progression to the pro-B cell developmental stage, is correlated with reduced numbers of pre-B cells. In addition, we observed altered distribution of cells within the pro-B cell fraction, as shown by the

reduced percent of $CD24^{high}$ cell in aged mice (Table 1). Van der Put et al. (91) recently reported that in aged mice that have a severe loss of pre-B cells the percentages of pro-B cells in Fr B and C/C' are lower as compared to young mice. These observations indicate that developmental progression within the pro-B cell stage is impaired in aged mice.

Together these observations indicate that impaired development within the pro-B cell stage and reduced generation of pre-B cells contributes to the reduced number of pre-B cells in aged mice. This is consistent with our hypothesis that reduced *rag2* expression and V(D)J recombinase activity contribute to reduced generation of the Ig heavy chains, pre-BCR expression, and progression to the pre-B cell stage of development.

Changes in the bone marrow microenvironment are sufficient to cause the age-related defects in B cell development.

One of our aims was to determine if defects in B cell development in aged mice was due to changes in the bone marrow hematopoietic microenvironment or to changes in the developing precursor cells. We performed complementary adoptive transfer experiments to separately evaluate the impact of the aged bone marrow microenvironment and the impact of aged precursor cells.

When bone marrow from young mice was transferred into mice of different ages we found that the aged bone marrow microenvironment was sufficient to produce reduced *rag2* expression and V(D)J recombinase activity in pro-B cells as well as reduced pre:pro ratio. In reciprocal experiments, we transferred bone marrow from mice of different ages into young hosts and found that *rag2* expression in pro-B cells and the pre:pro ratio were comparable regardless of donor age. These experiments indicate that the age-associated defects in *rag2* expression and V(D)J recombinase activity in pro-B cells and the pre:pro ratio are the result of changes in the bone marrow microenvironment rather than cell-intrinsic alterations. This does not rule out the existence of other cell-intrinsic alterations in B cell development.

Reduced pre-B cell numbers are due to impaired development not reduced numbers of pro-B cells.

Earlier studies indicate that the reduction in pre-B cells was not accompanied by a reduction in pro-B cells (101). However, our results, along with other recent papers, indicate that numbers of pro-B cells are significantly reduced in aged mice (91, 95, 96). The discrepancies are possibly due to differences in strains of mice, or the use of the monoclonal antibody AA4.1 to discriminate pro-B cells from other $CD43^{+}B220^{+}IgM^{-}$ cells. Recently it was proposed that the loss of pro-B cells in BALB/c is correlated with a severe loss of pre-B cells (91). The observation that numbers of pro-B cells are reduced in aged mice raised the concern that a smaller pool of pro-B cells might alone account for the reduction in numbers of pre-B cells. For this reason, we compared the number of pre-B cell between young and aged mice using both the absolute numbers of pre-B cells, and using the pre:pro ratio. By both measures the numbers of pre-B cells are reduced in aged mice, indicating that a reduction in the size of the pro-B cell pool does not account for the total reduction in pre-B cell numbers in aged mice. This is supported by the kinetic analysis conducted in the lab of Dr. Michael Cancro that indicates generation of pre-B cells is reduced in aged mice (personal communication, Dr. Michael Cancro [addressed in Chapter 4 Discussion]).

Potential mechanisms to account for alterations in the pro-B cell stage.

Factors and signals from bone marrow stromal cells are essential to the survival of developing B cell precursors and regulate the expression of transcription factors that govern commitment and differentiation of developing B cells. Cells at the pro-B cell stage require both cell-mediated contact and soluble factors. The stromal-derived factors IL-7, flt3L, SDF-1, and SCF are all critical for B cell development [as reviewed in (54)]. Specific extrinsic factors that induce *rag* expression have yet to be characterized. It is possible that the age-related decrease in *rag2* expression in pro-B cells could be due to attenuation of inductive signals.

Another possible mechanism that would contribute to reduced numbers of pre-B cells is decreased signaling through the pre-BCR due to reduced expression of □5 (95). λ5 is a component of the pre-BCR that is essential for developmental progression to the pro-B cell stage. Expression of surface and cytoplasmic □5 is reduced in pro-B cells of aged mice (95).

A third potential mechanism for the reduction in number of pre-B cells is reduced numbers of, or supportive capacity of stromal cells. We observed in aged mice that the percent of pro-B cells that express *rag2* is reduced; however, in pro-B cells that do express *rag2*, the per cell level of expression is similar to that of young mice. This could indicate that fewer pro-B cells receive signals and factors from the stromal microenvironment, but those that do receive the required factors develop normally. The alterations in pro-B cell development may be due to limited access of precursors to reduced numbers of stromal cells. Future experiments could be done to determine if stromal cell numbers are reduced in aged mice.

Reduced rag expression may contribute to restriction of the BCR repertoire.

Our results indicate that the pro-B cell stage of development is impaired in aged mice, resulting in reduced numbers of pre-B cells. This could contribute to reduced diversity of IgH chains, and the Ig repertoire in aged mice. Previous work has shown that receptor diversity is reduced in aged mice and that adaptive immunity is severely impaired. Our observations of reduced *rag2* expression and V(D)J recombinase activity support a model in which loss of IgH chain diversity results in reduced generation of mature naive B cells with novel B cell receptor specificity and thus reduced humoral adaptive immunity in aged mice.

Our results show that the documented reduction in pre-B cells occurs simultaneously with changes that occur in the pro-B cell stage of development that include reduced *rag2* expression and V(D)J recombinase activity. Alterations in the aged bone marrow

microenvironment are sufficient to produce these defects independent of cell-intrinsic alterations. Our work indicates that the loss of *rag2* expression and reduced V(D)J recombinase activity in pro-B cells and reduced numbers of pre-B cells are due to changes in the aged bone marrow microenvironment. The nature of these changes remains to be determined.

The alterations in B cell development in aged mice have similarities to those observed in aged humans. A better understanding of the underlying mechanisms responsible for these alterations would enable the design of experiments to address these changes in humans. Ultimately, this could lead to therapeutics that restore B cell production and thus the adaptive immune response to both pathogens and vaccines in aged humans.

CHAPTER III

Bone Marrow B Cell Populations in Aged Mice Are Influenced by Genetic Polymorphisms and Associated With Changes in T Cell Subsets

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INTRODUCTION

In both humans and mice, the immune system declines with age and is characterized by attenuated adaptive immunity, especially in response to new pathogens. Cellular and humoral immunity are both affected. Changes in cellular immunity include an increase in the percent of CD4 and CD8 T cells of the memory phenotype (103, 127-129) and an increase in the percent of T cells that display defects in activation (12, 103). T cell development also declines: beginning at maturity, the thymus decreases in size with age. In addition, altered T cell selection results in a skewed T cell repertoire (13, 130, 131). Changes in humoral immunity include a restricted immunoglobulin repertoire, reduced antibody production in response to pathogens and vaccines, and an increase in auto-reactive B cells (3, 113). B cell production is also impaired in aged mice. Compared to young mice, the size of the peripheral mature B cell pool is similar, but the rate of turn-over is greatly reduced and the rate at which cells enter the pool is lower (9).

During B cell development pluripotent hematopoietic progenitors progress through an ordered series of stages and develop into mature B cells. The stages of B cell development are defined by acquired characteristics that include expression of lineage-specific genes, immunoglobulin (Ig) gene rearrangement and expression of cell surface antigens (18, 123). As precursor B cells develop in the bone marrow they progress from hematopoietic stems cells through Common Lymphoid Progenitors (CLP), pro-B ($B220^{+}CD43^{+}IgM^{-}$), pre-B ($B220^{+}CD43^{+}IgM^{-}$), and immature B cell ($B220^{+}CD43^{-}IgM^{+}$) stages of development prior to export to the spleen (18, 123).

The most pronounced change in B cell development in aged mice is the reduction in numbers of pre-B cells, as aged mice can have as few as 10% of the number of pre-B cells found in young mice (90, 91, 95, 97, 101, 132, 133), this is also observed in Chapter 2. There is also an age-associated reduction in numbers of pro-B cells (91, 96). In contrast, the number of re-circulated mature B cells in the bone marrow initially increases with age (9, 91, 132) but declines as the age-related defects in B cell development become progressively worse with age (91). The mechanism(s) underlying these alterations in aged mice are unknown and may be multi-factorial.

Reduced transition of cells from the pro-B to pre-B cell stage of development may be critical to the reduced number of pre-B cells in aged mice. The developmental checkpoint between the pro- and pre-B cell stages requires assembly and signaling through the pre-BCR composed of the Ig heavy chain (IgH), λ5 and VpreB. Synthesis of the IgH chain occurs in the pro-B cell stage of development and requires expression of *rag1*and *rag2* to mediate V(D)J recombination of IgH genes. We have found that *rag2* expression is reduced within pro-B cells in aged mice and this reduction is correlated with both a reduction in V(D)J recombinase activity and diminished numbers of pre-B cells (Chapter 2 and Labrie et al. in preparation). A second mechanism to account for reduced numbers of pre-B cells is the age-related reduction in λ5 expression (95, 100). Reduced λ5 may limit the efficiency with which the pre-BCR transmits signals required

for progression to the pre-B cell stage. Both findings point to a limitation at the pro-B to pre-B cell transition.

One approach to understanding the mechanism(s) responsible for diminished B cell generation with age is to identify specific genes that influence B cell development in an age-dependent manner. As a first step towards this goal, we proposed that genetic polymorphisms in different mouse strains might influence the pace at which age-specific alterations in B cell development occur with advancing age and/or the severity of these changes. These polymorphisms could occur in genes that are expressed in developing B cells, in bone marrow stromal cells required to support B cell generation, or alternately in peripheral T cells that in turn might influence B cell development. As an initial step in characterizing the genetic influences on age-sensitive B cell developmental pathways, we used a genome scan approach to determine if polymorphisms between different mouse strains are associated with age-related changes in B cell development in a large cohort of genetically heterogeneous mice.

This cohort (termed UM-HET3) was produced by mating BALB/cJ x C57BL/6J F1 females and C3H/HeJ x DBA/2J F1 males. The four progenitor inbred strains were chosen for several reasons, including their relatively substantial interstrain genetic variation (105). Variation at simple sequence length polymorphism sequences (SSLP) have been successfully used to identify QTL associated with lifespan (Jackson et al., 2002), age-related T cell phenotypes (Jackson et al., 1999; Miller et al., 2003), frequency of splenic B cells (134), and other quantitative phenotypes (135). We expected this cohort to display diversity with respect to age-related alterations in B cell development and thus enable us to determine if genetic polymorphisms between these strains are associated with specific differences in the age-related changes in B cell development.

We also wanted to determine if correlations exist in the aged cohort between pre-B cell frequencies and other lymphocyte populations. The use of aged genetically

heterogeneous mice enabled us to determine if altered frequencies of B cell precursor subsets were correlated with age-associated increases in mature re-circulated B cells in the bone marrow or with age-associated alterations in peripheral T cell subsets.

Here we report that the frequencies of both pre-B cells and re-circulated bone marrow B cells in aged mice are linked to specific genetic polymorphisms. In addition, we show that the early stages of B cell development, pro- and pre-B cells, are not correlated with the frequency of peripheral memory and anergic T cells subsets nor mature re-circulated B cells in the bone marrow. Instead, we found that pro- and pre-B cell frequencies were inversely correlated with the frequency of $B220^{int}IgM^{lo}$ cells in the bone marrow. Furthermore, we found that the frequencies of both re-circulated B cells and $B220^{int}IgM^{lo}$ cells are correlated with the frequencies of anergic and memory peripheral T cell subsets. These observations indicate that it is feasible to identify polymorphisms in mice that influence the effect of aging on B cell development. In addition, this work furthers our understanding of the relationships between peripheral T and B cells and B cell development in aged mice. The extension of these observations may lead to better understanding of the genetic basis of age-related changes in B cell development.

MATERIALS AND METHODS

Mice generation and husbandry (conducted at University of Michigan)

This study utilized a genetically heterogeneous mouse population, denoted UM-HET3 that was derived from four inbred strains: BALB/cJ(C), C57BL/6J(B6), C3H/HeJ(C3), and DBA/2J(D2). The experimental animals were the female progeny of (C x B6)F_1 females and (C3 x D2)F_1 males. This breeding scheme produces mice that are genetically distinct but can be considered full siblings. The F_1 breeding animals were purchased from the Jackson Laboratories (Bar Harbor, ME). Throughout the study, all mice were housed in a single suite of specific-pathogen free (SPF) rooms under identical environmental conditions (12:12 hour light:dark cycle, 23°C) and given ad libitum access to water and laboratory mouse chow. Quarterly tests of sentinel mice showed that the facility remained SPF throughout the study (136). Blood samples were obtained by venipuncture at ages 4, 12, and 15 months. At six months of age an abdominal skin biopsy was obtained under brief methoxyfluorane anesthesia. In addition, a tail clip was taken at 1 to 2 month for DNA analysis. The animals were humanely euthanized at 18 months of age. Bone marrow specimens were removed by flushing from tibias, and sent by overnight courier from the University of Michigan to the University of Massachusetts for analysis.

Genotyping (conducted at University of Michigan)

Genotyping was performed by standard polymerase chain reaction (PCR) amplification of genomic DNA from each animal. The PCR amplified DNA was genotyped using an ALFexpress automated sequence analyzer (Pharmacia, Piscataway, NJ); the details of this genotyping method have been described previously (7). Primer pairs were purchased from MWG Biotech Inc. (High Point, NC). In total, 185 markers were examined from 99 genetic loci. Of the 99 loci, 86 markers were informative for both the maternal and paternal derived alleles and 13 loci were only informative for maternal or paternal. Genetic loci were initially selected using data provided by the Mouse SSLP Database, Whitehead/MIT Center for Genome Research (137); Cambridge, MA;

http://www.genome.wi.mit.edu/cgi-bin/mouse/) or the Mouse Genome Database 3.1, Mouse Genome Informatics, the Jackson Laboratory (http://www.informatics.jax.org/). Loci were chosen that were polymorphic among the four inbred strains and were distributed across the genome (15 to 20 cM intervals). The final 99 loci were selected based on their ability to be amplified by PCR and viewed on the gel electrophoresis system reproducibly and easily. Chromosomal localization and order of markers were calculated using the MapManager QTX program package (138).

Statistical analysis (conducted at University of Michigan)

A single point genome-wide search was performed for each trait in order to detect QTLs that may be associated with the trait. To make the analysis consistent for all partially and fully informative markers, 4-way informative markers were split into two sets of bi-allelic markers, informative for either the maternally or paternally transmitted alleles. One-way analysis of variance models, with a trait as the dependent variable and a bi-allelic marker as the factor with two levels, were used to perform genome-wide searches for all 185 bi-allelic markers. The strength of linkage associations between genetic markers and mechanical traits was evaluated using a permutation-based test of statistical significance. This test generates an "experimentwise" acceptance criterion to take into account the multiple hypotheses that were tested in this search and to avoid type I error inflation (139). A null distribution for permutation analysis was generated based on 1000 shuffles of original phenotype data. An experimentwise $p \leq 0.05$ was considered statistically significant. The percent of variance in each mechanical trait that can be explained by genetic effects was estimated in a standard way from corresponding regression models.

FACS analysis of bone marrow (conducted at University of Massachusetts)

Cells were suspended in staining media [RPMI 1640 deficient for biotin, flavin, and phenol red (Irvine Scientific, cat # 9826, Santa Ana, CA), with 10 mM HEPES pH 7.2, 0.02% sodium azide, 1 mM EDTA and 3% newborn calf serum]. Cells were washed and suspended at 3 x 10^7/ml in staining medium and treated for 10 minutes on ice with purified antibody (2.4G2) to block Fc receptors. Cells were incubated with primary antibodies for 20 minutes followed by three washes, then incubated with streptavidin-Cy5 -PE for 15 minutes, followed by three washes. Primary antibodies were anti-IgM-FITC, anti-CD43-PE, and anti-B220-Biotin. Cells were re-suspended in staining medium with propidium iodide (1μg /ml) to exclude dead cells. Flow cytometry was performed on a FACSCaliber (BDIS). Dead cells were excluded from analysis thought the use of propidium iodide. Data was analyzed with FlowJo Software (Tree Star, Inc).

FACS analysis of T cell subsets (conducted at University of Michigan)

Two color flow cytometry analyses were conducted as previously described (140) on samples of peripheral venous blood obtained at 8 and then again at 18 months of age. Table 5 shows the definition of the four T cell subsets that were reported in this study.

TABLE 5. T Cell Subsets Tested in 8- and 18-Month Old UM-HET3 Mice

Subset Designation	Description	Calculation
CD4M	Memory CD4 cells	$CD4^{+}$,$CD44^{high}$ as % of CD4
CD8M	Memory CD8 cells	$CD8^{+}$,$CD44^{high}$ as % of CD8
CD4P	P-glycoprotein-positive CD4 cells	$CD4^{+}$, R123-extruding as % of CD4
CD8P	P-glycoprotein-positive CD8 cells	$CD8^{+}$, R123-extruding as % of CD8

Measurement of T cell proliferation (conducted at University of Michigan)

Total numbers of nucleated cells were counted at necropsy using a hemocytometer. Mice with total spleen cell numbers less than 1.5×10^8 were included in analysis of spleen cellularity and T cell functional outcomes.

Immunizations and antibody response (conducted at University of Michigan)

Mice were immunized with 5×10^8 sheep erythrocytes in 0.2 ml of saline at 4 months of age, and with 10^9 turkey erythrocytes at 15 months of age. Serum was obtained by tail venipuncture at 2 weeks after immunization and assayed for anti-erythrocyte antibody titer by hemagglutination starting with a 1:16 serum dilution.

Exclusion criteria

Bone marrow analyses were conducted on 275 mice. Of these, 224 had genotypic data available for *D19Mit41* and 203 had genotypic data available for marker *D15Mit100*. Missing values are the result of errors in DNA preparation or ambiguous typing results.

RESULTS

Analyses of bone marrow B cell populations in aged UM-HET3 mice

A cohort of 275 female UM-HET3 mice derived from mating BALB/cJ x C57BL/6J F1 females and C3H/HeJ x DBA/2J F1 males was generated and aged. DNA was obtained from each mouse for genotyping and used to evaluate 87 SSLPs, 77 of which discriminate between all four parental strains. SSLPs were revealed by PCR that detects strain differences documented in the Mouse Simple Sequence Length Polymorphism Database, Whitehead/MIT Center for Genome Research (carbon.wi.mit.edu:8000/ftp /distribution/mouse_sslp_releases/may99) (137). Mice were immunized at four months of age with sheep red blood cells and at 15 months of age with turkey red blood cells as part of a separate study. At 18 months of age mice were euthanized and evaluated for several aspects of T and B cell immune status. We used FACS analysis to determine the frequencies of pro-B ($IgM^-B220^+CD43^+$) and pre-B cells ($IgM^-B220^+CD43^-$) in the bone marrow of UM-HET3 mice (Figure 6). Both pro- and pre-B cell frequencies were normally distributed among the 275 mice tested (data not shown).

FIGURE 6. Flow cytometric analysis of B cell subsets in bone marrow of aged UM-HET3 mice.

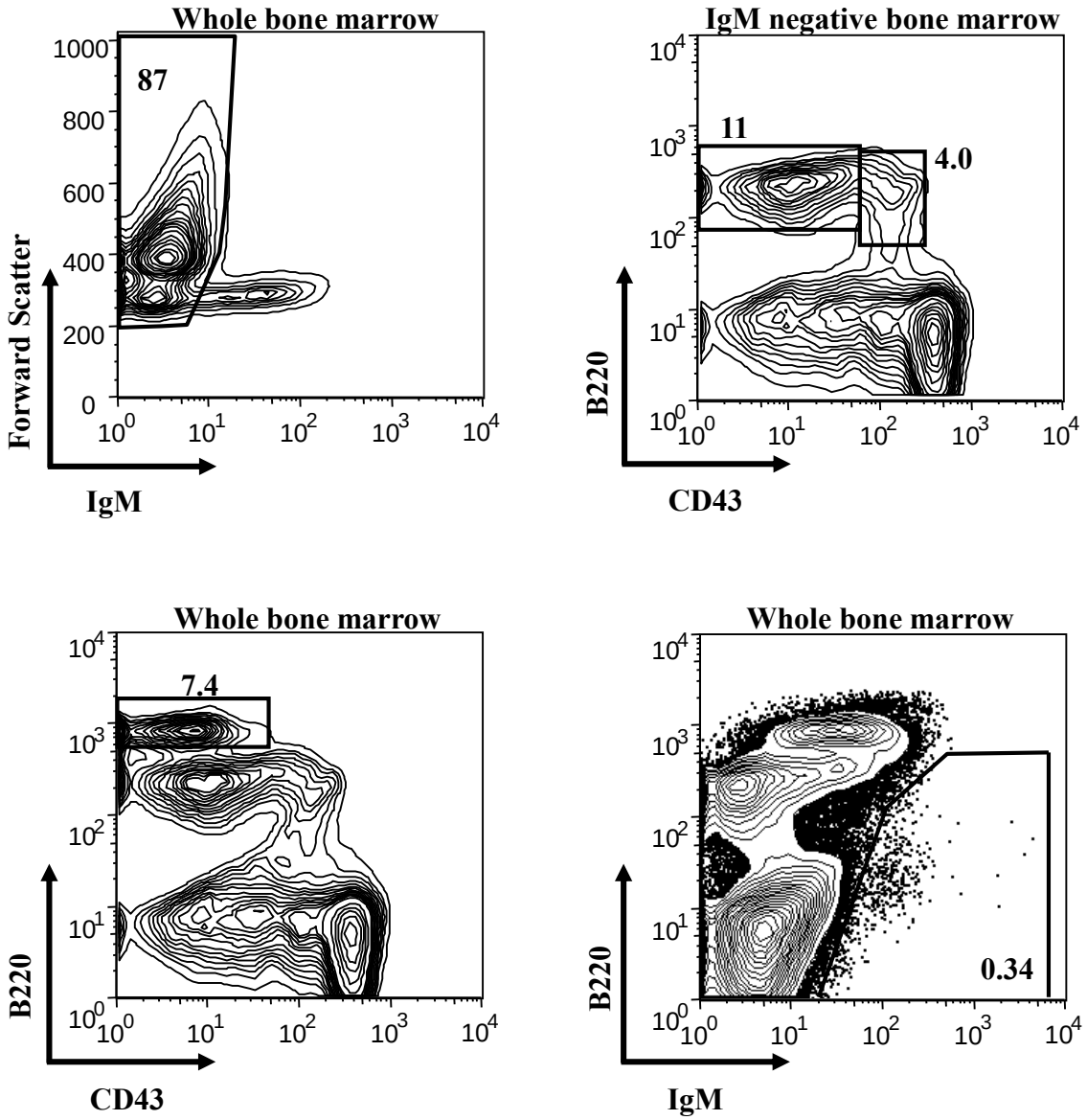


FIGURE 6. Flow cytometric analysis of B cell subsets in bone marrow of aged UM-HET3 mice. Bone marrow from a representative aged UM-HET3 mouse was stained with antibodies to IgM, B220 and CD43 for flow cytometric analysis. Labels above each graph indicate the type of cells displayed. The number within or near each gate reflects the percent of cells within the gate. The upper left panel depicts forward scatter and IgM expression in whole bone marrow. The upper right panel depicts detection of pro-B ($IgM^-B220^+CD43^+$) and pre-B ($IgM^-B220^+CD43^-$) cells in the IgM^- subset. The lower left panel depicts detection of $B220^{high}CD43^-$ re-circulating B cells in whole bone marrow. The lower right panel depicts detection of $B220^{int}IgM^+$ cells in whole bone marrow.

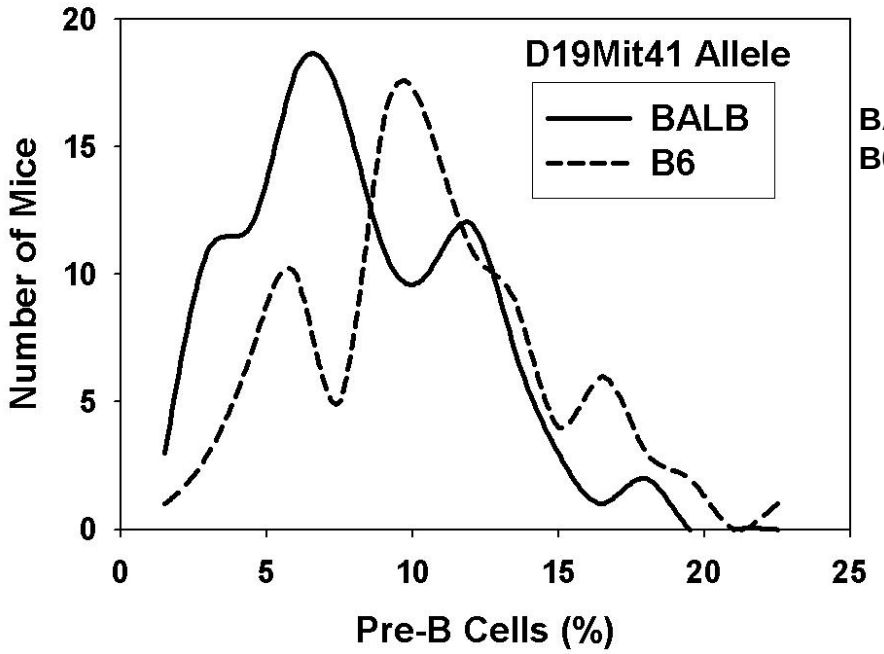
We also measured the frequency of mature re-circulated B cells, as estimated by the fraction of cells with the $B220^{high}CD43^{-}$ phenotype (see Figure 6, lower left panel); the frequency of these cells increases in aged mice (9, 91, 132). We also noticed that a subset of aged mice had an increased frequency of $B220^{int}IgM^{+}$ cells in their bone marrow. The mean frequency of $B220^{int}IgM^{+}$ cells in the marrow of aged UM-HET3 mice was 1.1% (see Figure 6, lower right panel for an example) and eight mice had frequencies exceeding 3%. These cells are rare in un-immunized young mice and have characteristics of early stage memory cells (141). Because the distribution of the $B220^{int}IgM^{+}$ subset was not normal, we used the log of the number of $B220^{int}IgM^{+}$ cells, which was normally distributed, for our statistical analyses.

QTL Analysis of B cell subsets

A genome scan was used to determine if genetic polymorphisms among the four inbred grandparental strains of mice influenced the frequency of B cell subsets in the bone marrow of aged UM-HET3 animals. Two quantitative trait loci reached experimentwise significance levels, and these are listed in Table 6. We found that the frequency of pre-B cells is modulated by a locus linked to SSLP marker *D19Mit41* on chromosome 19 with $p = 0.004$. Of 95 mice that inherited the C57BL/6J SSLP at *D19Mit41*, the frequency of pre-B cells was $9.8 \pm 4.3\%$ (mean ± SD). Of 108 mice that inherited the BALB/cJ SSLP, the frequency of pre-B cells was $7.4 \pm 3.8\%$. Histograms depicting the frequency of pre-B cells in mice differing in their *D19Mit41* allele are shown in Figure 7.

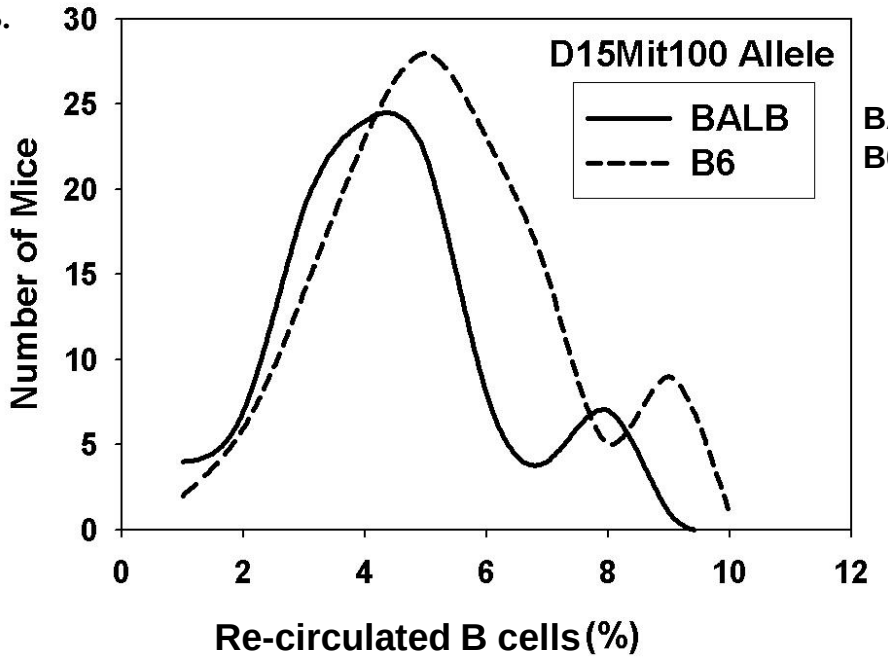
FIGURE 7. Correlations of genomic markers polymorphic between C57BL/6J or BALB/cJ mice with the frequency of pre-B cells (IgM^- $B220^+CD43^-$) and the frequency of re-circulated B cells ($B220^{hi}CD43^-$) in the UM-HET3 aged mouse cohort

A.



BALB 7.4 ± 3.8%
B6 9.8 ± 4.3%

B.



BALB 3.9 ± 1.8%
B6 4.8 ± 1.8%

FIGURE 7. Correlations of genomic markers polymorphic between C57BL/6J or BALB/cJ mice with the frequency of pre-B cells ($IgM^{-}B220^{+}CD43^{-}$) and the frequency of re-circulated B cells ($B220^{hi}CD43^{-}$) in the UM-HET3 aged mouse cohort. (A) D19Mit41 is correlated with the frequency of pre-B cells. The distribution, mean and standard deviation of the percent of pre-B cells in whole bone marrow are shown for aged mice which tested positive for either the C57BL/6J or BALB/cJ specific allele of the D19Mit41 SSLP. (B) D15Mit100 is correlated with the frequency of re-circulated B cells. The distribution, mean and standard deviation of the percent of $B220^{hi}CD43^{-}$ cells in bone marrow are shown for aged mice which tested positive for either the C57BL/6J or BALB/cJ specific allele of the D15Mit100 SSLP.

TABLE 6. Associations between frequencies of B cell subsets in the bone marrow and genetic markers.

The table shows B cell developmental subsets and correlated genomic markers; (a) position from centromere in centimorgans; (b) position from centromere in millions of base pairs; (c) experiment-wise p-value from permutation test; (d) percentage of phenotypic variation attributable to the allelic difference indicated.

Trait	Marker	Position (cM) (a)	Position (mpb) (b)	P< (c)	Allele order	% Effect (d)
Pre-B	D19Mit41	16	18	0.004	B6 > BALB/c	8.5%
$B220^{high}CD43^{-}$	D15Mit100	21	51	0.03	B6 > BALB/c	5.7%

The frequency of mature re-circulated B cells was influenced by a gene on chromosome 15 linked to the SSLP marker *D15Mit100* at a significance level $p < 0.03$ (Table 6). Of the 127 mice that inherited the C57BL/6J SSLP at *D15Mit100*, the frequency of re-circulated cells was 4.8 $\pm$ 1.8%. Of the 97 mice that inherited the BALB/cJ SSLP, the frequency of re-circulated B cells was 3.9 $\pm$ 1.8%. Histograms depicting the frequency of re-circulated B cells based on inheritance of the D15Mit100 genomic marker are shown in Figure 7.

Parallel analyses found no significant evidence for QTL modulating the frequencies of pro-B cells and $B220^{int}IgM^{+}$ cells in this mouse population.

Frequencies of pro- and pre-B cells are not correlated with peripheral T cell subsets, but they are inversely correlated with the frequency of $B220^{int}IgM^{+}$ cells.

Analysis of the UMHET-3 aged cohort also afforded the opportunity to determine whether correlations exist among the various age-sensitive B cell subsets and indices of T cell immune status including T cell subset levels and hemagglutination results. We therefore, as a preliminary exploration, examined a correlation matrix to seek evidence for relationships that might deserve further attention in later studies. Table 7 collects evidence for correlations among the B cell subsets, and also includes information on correlations with antibody responses. Mice with high levels of pro-B cells tended to have high levels of pre-B cells and low levels of the $B220^{int}IgM^{+}$ subset. High pro-B cell levels, at 18 months of age, were also characteristic of mice that had been able to produce high titer anti-erythrocyte antibody at 4 months of age (Table 7), although no such correlation was seen when antibody responses were evaluated at 15 months using turkey erythrocytes.

TABLE 7. Correlation Coefficients pro- and pre-B cells.

The table shows correlations among pro-B, pre-B and $B220^{int}IgM^{+}$ cells and antibody response to immunization with sheep RBC. N = 208-216 mice for each calculation, with pairwise exclusion for missing data. Approximately 40 mice were excluded because of splenomegaly (spleen cell number > 150 x 10^6), but similar correlations were noted when these mice were included in the calculations.

PRO-B	r = 0.37 (p < 0.0001)	r = -0.22 (p = 0.0028)	r= 0.21 (p=0.0023
r = 0.37 (p < 0.0001)	**PRE-B**	-0.27 (p < 0.0002)	r = 0.01 (p = 0.84)
r = -0.22 (p = 0.0028)	-0.27 (p < 0.0002)	**Log $B220^{int}IgM^{+}$**	r = 0.05 (p = 0.45)
r= 0.21 (p=0.0023	r = 0.01 (p = 0.84)	r = 0.05 (p = 0.45)	**Anti-sheep hemagglutination**

Table 8 reports statistics relating B cell subsets to four T cell subsets that rise dramatically with age, both in blood and in spleen. Three of these subsets (the memory subsets CD4M and CD8M, and the anergic P-glycoprotein positive subset CD4P, measured in peripheral blood) have, in previous work, been shown to predict remaining lifespan (142, 143) and thus may serve as biomarkers of aging in the immune system. Thus it is noteworthy that the $B220^{int}IgM^{+}$ B cell population is positively correlated to CD4M, CD8M and CD4P in both blood and spleen; five of these six correlations are statistically significant at $p < 0.02$. The CD8P subset also shows a positive correlation for blood T cells, though not for spleen T cells. Thus high levels of the $B220^{int}IgM^{+}$ subset are seen characteristically in mice whose T cell immune systems resemble those of older mice. The correlations with the $B220^{hi}CD43^{-}$ subset are somewhat less consistent. There are significant negative correlations with the CD4P, CD8P and CD4M subsets in blood, and with splenic CD4P cells, suggesting that mice with more advanced T cell aging tend to have lower levels of marrow $B220^{hi}CD43^{-}$ cells, but the correlations with the other four tabulated T cell values are unimpressive. We found no significant correlations between any of these T cell subsets and the pre-B or pro-B levels in bone marrow.

TABLE 8. Correlations between T and B cell subsets.

Each entry shows the Pearson correlation coefficient between the indicated B cell subset (left column) and age-sensitive T cell subset (column headers), together with the corresponding significance level. For example, the upper left entry shows R = -0.16 and $p < 0.01$ for the relationship between $B220^{high}CD43^{-}$ marrow B cells and CD4P blood T cells. Boldface highlights entries for which $p < 0.05$. N = 254 - 263.

BLOOD				
	CD4P	CD8P	CD4M	CD8M
$B220^{high}CD43^{-}$	**-0.16 (0.01)**	**-0.19 (0.002)**	**-0.18 (0.004)**	-0.10 (0.1)
$B220^{int}IgM^{+}$	0.09 (0.11)	**0.14 (0.02)**	**0.39 (0.001)**	**0.23 (0.001)**
SPLEEN				
	CD4P	CD8P	CD4M	CD8M
$B220^{high}CD43^{-}$	**-0.20 (0.001)**	0.01 (0.8)	-0.02 (0.8)	-0.01 ((0.8)
$B220^{int}IgM^{+}$	**0.18 (0.003)**	0.08 (0.2)	**0.46 (0.001)**	**0.24 (0.001)**

We also evaluated the relationship of these two B cell subsets to splenic T cell function as estimated by *in vitro* proliferation assays. Mice with high levels of the $B220^{int}IgM^{+}$ subset tended to show high proliferation in responses to anti-CD3 antibody, with or without added anti-CD28 antibody ($p < 0.005$ in each case), but did not show significantly higher responses to Con A (data not shown); these correlations may simply reflect the ability of anti-CD3 antibody to stimulate proliferation of memory T cells, which tend to be higher (Table 8) in mice with high levels of the $B220^{int}IgM^{+}$ subset.

DISCUSSION

One of the key alterations in B cell development in aging mice is the loss of pre-B cells. The extent to which genetic polymorphisms contribute to these age-related changes was previously unknown. Therefore, we undertook a QTL analysis of B cell development in a cohort of aged genetically heterogeneous mice. In addition, a number of changes occur in murine lymphocyte populations with age, although it was not known if these changes occur independently but parallel to each other or if they are interrelated and occur as part of an overall immunosenescence or immunomodulation. For example, thymus involution is parallel to the decline in B cell development, but it is not known if these processes are causally related. Analysis of correlations between these age-related changes in a heterogeneous population of mice was undertaken to identify which changes occur in conjunction with each other and begin to understand relationships among these age-related traits.

Polymorphism on chromosome 19

We found that the frequency of pre-B cells in the bone marrow is influenced by a polymorphism linked to SSLP marker *D19Mit41* on chromosome 19. The frequency and number of pre-B cells are reduced in aged mice. Various mechanisms to account for this reduction have been proposed, including reduced generation of pre-B cells, increased apoptosis of pre-B cells and increased rate of maturation. We and others have found that development within the pro-B cells stage is impaired in aged mice and that fewer pre-B cells are produced (9, 90, 91, 96). In the cohort of aged mice, the frequency of pre-B cells was influenced by a polymorphism between BALB/cJ and C57BL/6J mice located on chromosome 19, linked to the *D19Mit41*. The genomic scan is not of sufficient resolution to provide useful information about which specific genes, linked to *D19Mit41*, might be responsible for this phenotype. It is tempting to speculate that this genetic difference has an impact on developmental progression of pro-B cells. The gene on chromosome 19 that influences the frequency of pre-B cells in aged bone marrow could be expressed in either B cell precursors or in stromal cells that provide essential support for B cell development. Stromal cells from aged mice are less supportive of B cell

development than similar cells harvested from young mice (93, 94, 101). In addition, we have found that the decline in pre-B cell frequency, *rag2* expression and V(D)J recombinase activity in aged mice are all conferred by the aged bone marrow microenvironment (as shown in Chapter 2).

Polymorphism on chromosome 15

The frequency of mature re-circulated B cells in the bone marrow in the aged mouse cohort is influenced by a polymorphism linked to SSLP marker *D15Mit100* on chromosome 15. Re-circulating bone marrow B cells are mature B cells that resemble spleen follicular B cells and the B cells found in lymph nodes and peripheral blood. These cells are presumably an important part of immune surveillance and decline of this population with age might underlie or reflect compromised mature B cell function. A detailed understanding of requirements for B cell trafficking to bone marrow does not exist. However, it is tempting to speculate that this polymorphism could occur in a gene involved in B cell homing, either expressed in B cells or in the bone marrow microenvironment.

In the case of both polymorphisms (*D15Mit100* and *D19Mit41),* the impact associated with the polymorphism could be age-dependent or age-independent. In this study a QTL analysis was conducted on 18 month old mice. Bone marrow sampling is a terminal assay and for this reason it was not possible to obtain samples from these mice at more than one age, although in future work it should be possible to evaluate effects of these two QTL on bone marrow B cells of young UM-HET3 mice. Age-independent genomic differences between mice could lead to identification of genes that regulate B cell development that are currently unknown. Age-sensitive genomic differences have the potential to increase our understanding of mechanisms of immunosenescence in mice.

Correlations

Age-associated changes in B cell development occur concurrent with changes in peripheral T cell subsets. We assessed relationships between B cell subsets and

peripheral T cell subsets in the aged UM-HET3 cohort to determine if age-associated changes were related to each other, to seek initial indications of potential causal relationships or common underlying mechanisms. We found that the frequencies of pro- and pre-B cells in the bone marrow are themselves correlated ($r= 0.37$, $p < 0.0001$; Table 7) in the UM-HET3 aged cohort. This correlation is not surprising, as pro-B cells are precursors of pre-B cells. We have also observed a significant correlation between the frequencies of pro-B and pre-B cells in other experiments involving aged, inbred mice.

In addition to a decline in the number of pre-B cells in aged mice, there is also an increase in the number of mature re-circulated B cells in the bone marrow (9, 91, 132). The requirements for homing, survival and persistence of mature B cells in the bone marrow are not well understood. It is possible that the increased number of re-circulated B cells in the bone marrow competes with developing precursors, therefore resulting in attenuated B cell development. If this were true, we would expect there to be an inverse correlation between frequencies of pre-B cells and re-circulated B cells in the bone marrow. However, we did not observe a correlation between re-circulated B cells and pre-B cell frequencies in the UM-HET3 aged cohort. This could indicate that mature B cells do not inhibit earlier stages of B cell development or that this effect is not seen as early as 18 months of age. Analysis of pre-B cells and re-circulated B cells in bone marrow in inbred strains of mice indicate that the "aged phenotype" occurs more frequently in mice older than 18 months of age.

Another age-related change in the immune system is thymic involution, which begins shortly after adolescence. While B cell development is generally regarded as independent of T cells, pre-B cell numbers are lower in athymic mice than in age-matched controls and are partially restored following transfer of syngeneic T cells (97, 98). In addition, numbers of pre-B cells are increased in aged and athymic mice following injection of supernatant harvested from cultures of CD8+ T cells activated *in vitro* or rIL-16 (97, 98). These observations support the concept that reduced thymic

output of T cells or altered peripheral T cell populations could contribute to lower pre-B cell numbers in aged mice. If this idea is correct, mice with more profound age-related alterations in T cell subsets or activation might be expected to suffer greater impairment of B cell development. We evaluated pre-B and pro-B cell numbers in mice differing in the frequencies of peripheral T cells subsets known to be increased in aged mice (CD4M, CD8M, CD4P and CD8P in blood and spleen). We also measured activation of splenic T cells following *in vitro* stimulation with anti-CD3 antibodies with or without anti-CD28 costimulation. We found no correlation between the frequencies of pre-B or pro-B cells in the bone marrow and any of the T cell subsets, and no correlation with proliferation of splenic T cells. This lack of correlation suggests that the age-associated reduction in pre-B cells occur independent of alterations in T cell subsets in aged mice.

Interestingly, the age-sensitive peripheral T cell subsets were, for the most part, negatively correlated with re-circulated B cells and positively correlated with $B220^{int}IgM^{+}$ B cells. The frequency of $B220^{int}IgM^{+}$ cells in the bone marrow was greater in mice that had more extensive age-associated changes in T cell subsets (higher frequencies of CD4M, CD8M and in both blood and spleen and higher CD4P in spleen, and CD8P in blood). The frequency of $B220^{int}IgM^{+}$ cells in the bone marrow was inversely correlated with the frequency of pro- and pre-B cells (Table 7). These $B220^{int}IgM^{+}$ cells could be pre-memory plasma cells that have homed to the bone marrow. Further studies would be needed to evaluate whether these cells were elicited in response to the immunization at 15 months with turkey RBC. The mice were housed in SPF conditions and quarterly serology testing of sentinel mice found no evidence for viral infection during the course of these experiments, but we cannot completely exclude that increased $B220^{int}IgM^{+}$ cells could result from encounter with other infectious agents or environmental antigens of unknown kinds. These cells might also be activated autoimmune B cells. There is an increase in low affinity auto-reactive antibodies in aged mice (2, 3, 144, 145). It is possible that advanced T cell decline with age leads to a reduction in tolerance to self-antigens, resulting in an increase in activated autoimmune B

cells. The relationship of increased frequency of $B220^{int}IgM^{+}$ cells in the bone marrow and autoimmunity deserves further study.

In the UM-HET3 cohort, mice with higher frequencies of $B220^{hi}CD43^{-}$ re-circulated B cells in the bone marrow had lower percentages of memory CD4 and CD8 and anergic CD4 (i.e. CD4P) cells in blood, and low levels of CD4P cells in spleen (Table 8). These observations could indicate that CD4M, CD8M and CD4P cells inhibit production or homing of re-circulated B cells to the bone marrow. The frequency of re-circulated B cells in the bone marrow initially increases in aged mice (9, 91, 132). However, B cell development defects are more extreme at later ages and in mice with severe losses of pre-B cells (defined as less than 20% of that seen in young mice) also have reduced numbers of re-circulated B cells in the bone marrow (91). It is possible that in these mice the frequency of re-circulated B cells in the bone marrow is reduced because the production of new cells to replace those that are lost is very limited. It would be of interest to determine if the correlation between re-circulated B cells and memory and anergic T cells is still observed in those conditions.

Summary

In this work, we show that genetic polymorphisms affect the frequencies of pre-B and re-circulated B cells in aged mice. The refinement of the QTL analysis using a higher density of genomic markers and other genetic approaches should permit the identification of genes that modulate B cell development in aging. We also show that the frequencies of pro- and pre-B cells do not appear to be directly impacted by frequencies of peripheral T cells or re-circulated B cells in the bone marrow. However, pro- and pre-B cell frequencies are negatively correlated with the frequency of $B220^{int}IgM^{+}$ cells, a subset that is also positively correlated with peripheral T cell memory subsets. Furthermore, frequencies of mature re-circulated B cells in the bone marrow are negatively correlated with peripheral anergic T cells and CD4 memory T cells in blood. This data suggest the “aged immune phenotype” varies greatly in a genetically diverse

cohort of 18 month old mice. In addition, a more advanced aged phenotype might be indicated by high levels of anergic and memory T cells, high levels of bone marrow $B220^{int}IgM^{+}$ cells**,** and low levels of both pre-B cells and bone marrow re-circulated B cells.

CHAPTER IV

Discussion

4.1 At the start of this work, the mechanisms responsible for loss of pre-B cells in aged mice were unknown.

With age, the immune system in mice and human is attenuated and characterized by decreased ability to respond to pathogens, decreased tumor surveillance and increased incidence of autoimmunity (1, 2). Lymphocyte subsets change with age. The T cell population has an increased frequency of memory and anergic cells (1, 2). And mature B cell frequency and numbers increase in the bone marrow of aged mice (3, 9). Both B and T cell populations in aged mice are characterized by increased clonal expansion resulting in reduced immunogloblin and TCR receptor diversity (2, 3).

B cells development is reduced in aged mice, and one hallmark of this is a reduced number of pre-B cells (9, 90-92, 95, 96, 98, 99, 101, 113, 132). The mechanism of this decline in pre-B cells was not well understood. *rag* expression was found to be reduced in total bone marrow of aged mice (98, 99), however, it was not know if loss of *rag* expression occurred in pro-B cells. It was also not known if loss of *rag* expression occurs in only the mice with fewer pre-B cells, as there is considerable variability in loss of pre-B cells between individual aged mice. One of the goals of this work was to determine if *rag* expression is lower in pro-B cells of aged mice, and if reduced *rag* expression was correlated with a loss of V(D)J recombinase activity and the number of pre-B cells. These observations would suggest that reduced *rag* expression contributes to the age-associated reduction in pre-B cells. In addition, it was not known if the aged microenvironment or cell-intrinsic defects provide the mechanism responsible for loss of pre-B cells. Nor was if known if genetic differences between strains of mice influence the onset or severity of age-associated defects in B cell development.

4.2 Determination of factors related to loss of pre-B cells in aged mice.

Much of the previous research related to diminished B cell development in aged mice was centered on comparisons of individual traits between groups of young and aged mice. This approach was informative in identifying age-associated defects. However, there were only limited studies in which the relationships between multiple age-related traits were studied. This approach can be instrumental in suggesting causal relationships between different defects.

In this work, I compared the occurrence and severity of multiple physical traits in both young and aged mice to identify potential relationships between age-associated changes in B cell development and T cells subsets. Comparisons were made of *rag2* gene expression and V(D)J recombinase activity (Chapter 2), numbers of cells in B cell developmental subsets, and frequencies of peripheral T cell subsets (Chapter 3). In addition, the contribution of age-associated defects in the bone marrow microenvironment and cell-intrinsic defects were addressed through the use of adoptive transfer experiments (Chapter 2). Finally, QTL analyses were used to determine if genetic differences between strains contribute to age-associated defects in B cell development (Chapter 3).

4.3 Brief overview of findings

I found that *rag2* expression is lower in pro-B cells of aged mice, and is correlated with reduced V(D)J recombinase activity and reduced numbers of pre-B cells (Chapter 2). The aged bone marrow microenvironment is sufficient to produce these defects (Chapter 2). In addition, polymorphic differences between C57BL/6 and BALB/c mice, located on chromosomes 19 and 15, are associated with frequencies of pre-B and re-circulated B cells in the bone marrow of 18 month old mice (Chapter 3). This latter finding indicates that genetic differences between strains may contribute to the age-associated decline in B cell development.

Furthermore, I found that the reduced frequency of pre-B cells in aged mice is not correlated with the frequency of re-circulated mature B cells in the bone marrow, nor with frequencies of peripheral T cell subsets (Chapter 3). However, frequencies of pro- and pre-B cells are inversely correlated with the frequency of $B220^{int}IgM^{+}$ cells in the bone marrow. $B220^{int}IgM^{+}$ cell frequency was greater in mice that had more extensive age-associated changes in T cell subsets (higher frequencies of CD4 and CD8 memory in both blood and spleen and higher frequency of CD4 anergic in spleen, and CD8 anergic in blood) (Chapter 3).

4.4 Reduced *rag2* expression and V(D)J recombinase activity may contribute to reduced generation of pre-B cells in aged mice.

Before conducting this work, I considered it possible that the reduction in pre-B cells in aged mice is due to reduced generation of pre-B cells, increased apoptosis of pre-B cells or a decrease in the amount of time for pre-B cells to develop into immature B cells. I have shown that in aged mice there are developmental defects within the pro-B cells stage of development. Specifically, there is reduced expression of *rag2* and reduced V(D)J recombinase activity in pro-B cells of aged mice (Chapter 2). The reduction in *rag2* expression was correlated with reduced V(D)J recombinase activity in pro-B cells and numbers of pre-B cells, suggesting that reduced *rag* expression impairs B cell development at the pro-B cell stage in aged mice. These findings indicate that generation of pre-B cell may be reduced in aged mice.

It should be noted that the V(D)J recombination reporter substrate used in these experiments may actually underestimate the degree to which V(D)J recombinase activity is reduced at the endogenous Ig locus in aged mice. This VEX V(D)J recombination substrate reporter is likely to be more efficient than the endogenous loci for two reasons. First the reporter was constructed using RSSs of the optimal consensus sequence. This RSS consensus sequence is a more efficient substrate for RAG1 and RAG2 but is not actually used at many Ig gene segments. The murine D_H and J_H RSSs are not consensus

and are in fact far less efficient than those utilized on the substrate (146, 147). Secondly, the sequence of the Ig gene segment that is flanking the RSS (coding ends) makes a significant contribution to the efficiency of recombination (148). The coding ends of the VEX substrate contains optimal sequences for recombination. Thus, the defect in V(D)J recombination in pro-B cells from aged mice may be much greater than what the VEX substrate may indicate. Our lab has observed that the substrate is recombined in 25% of the pro-B cells of *ERAG* knock-out mice (compared to 90% of pro-B cells in wt mice), whereas recombination of the endogenous Ig locus is severely impaired (at least 5 to 10 fold) in the *ERAG* knock-out (21, 82).

Effective V(D)J recombination of Ig genes requires both expression and activity of all proteins required for V(D)J recombinase activity and accessibility to the Ig gene segments. I have shown that *rag2* expression and V(D)J recombinase activity are reduced in pro-B cells of aged mice. The H2-SVEX transgenic mice provide an indicator of V(D)J recombinase activity that is independent of Ig gene segment accessibility (82). Thus, this work indicates that V(D)J recombinase activity is reduced in age mice, but does not address whether an age-associated reduction in Ig gene accessibility might also occur in aged mice. It is possible that both activity and accessibility are reduced. Accessibility of 5' V families of the IgH locus are dependent upon IL-7 signaling (49). In aged mice, both production of IL-7 by the stromal cells and responsiveness to IL-7 by developing procurers are reduced (93). Based on these observations, one could predict that accessibility to Ig gene segments is reduced in aged mice, however a direct study of V_H region utilization prior to cellular selection based on BCR affinity has not been conducted. Thus the data presented in Chapter 2 indicating that *rag2* expression and V(D)J recombinase activity are reduced in aged mice, may understate the degree to which V(D)J recombination of Ig genes is reduced in aged mice.

4.5 Generation of pre-B cells is reduced in aged mice.

At the start of this work it was not known if fewer pre-B cells in aged mice were due to reduced generation. The loss of *rag2* expression and V(D)J recombinase in pro-B cells are indicative of defective development in the pro-B cell stage and support the concept that production is limited in aged mice. Furthermore, my work and that of Van der Put et al. (91) show that developmental progression within the pro-B cell stage is attenuated in aged mice. I have shown that the percent of pro-B cells that are $CD24^{high}$ is reduced in aged mice. Van der Put et al. showed that there are reduced numbers of pro-B cells in developmental Fractions B and C. The majority of Fr. C cells have assembled a VDJ_H gene and those that express a pre-BCR begin to undergo proliferation prior to maturation to the pre-B cell stage. Reduced progression into Fraction B and C is also observed in *rag1* and *rag2* KO mice (43, 44). The defect in developmental progression in aged mice and *rag* KO mice are similar, indicating that a loss of *rag* expression in aged mice could contribute to reduced generation of pre-B cells.

Another potential mechanism that could contribute to reduced generation of pre-B cells is reduced *λ5* expression in pro-B cells of aged mice (95). *λ5* and *VpreB* form the surrogate light chain, which together with the IgH chain, Igα and Igβ form the pre-BCR. Pre-BCR signaling is essential for the transition from pro- to pre-B cell stage of development.

While our observations and those of Sherwood (reduced λ5 expression) provide possible mechanisms that would account for reduced generation of pre-B cells, it is work from Dr. Michael Cancro that shows pre-B cell generation is reduced in aged mice. BRDU labeling experiments show that labeling rates of pro-B cells are similar in young and aged mice, indicating a renewal rate of 35-45%, and ~ 2 million cells per day (Table 9), however, labeling kinetics within the pre-B cell pools of young and aged mice differ significantly (Labrie et al. in preparation). Young adult mice generate 9 to 13 million pre-B cells daily and aged individuals produced 2 to 5 million pre-B cells per day (Table

9). The renewal rates of pre-B cells are similar in young and aged mice (32 to 42% per day) indicating that the residency time within the pre-B cell stage is unchanged with age (Labrie et al. in preparation). The four-fold decrease in generation of pre-B cells corresponds well with the degree of pre-B cell loss (loss of cell numbers) in aged mice and indicates that reduced generation of pre-B cells is most likely the major contributor to reduced pre-B cell numbers in aged mice.

TABLE 9. Production and renewal rates of marrow B cell subsets in Bone Marrow Chimeras determined from *in vivo* BrdU labeling

		Untreated Mice		Bone Marrow chimeras		
		Young	Aged	Young ⇨ Young	Young ⇨ Aged	Aged ⇨ Young
Pro	Number of cells (x 10^{-6})	3.5 ± 1.3	2.1 ± 1.3$^{++§}$	4.0 ±1.2	2.3 ± 1.3^{+}	3.2 ± 1.0
	Renewal Rate (% of pool/day)‡	35.8	34.1	31.9	33.0	38.5
	Production Rate (Cells/day x 10^{-6})‡	1.2	0.7^{+}	1.2	0.8	1.4
Pre	Number of cells (x 10^{-6})	28.0 ± 9.7	6.8 ± 4.3^{++}	33.3 ± 10.4	10.0 ± 5.9^{++}	30.4 ± 12.3
	Renewal Rate (% of pool/day)	37.3	32.0	38.0	31.7	35.8
	Production Rate (Cells/day x 10^{-6})	9.3	2.3^{++}	10.0	2.2^{++}	9.5
Immature	Number of cells (x 10^{-6})	9.3 ± 3.2	7.8 ± 4.1	10.9 ± 2.4	4.7 ± 3.5	9.1 ± 4.0
	Renewal Rate (% of pool/day)	20	11^{+}	23.0	17.1	20.7
	Production Rate (Cells/day x 10^{-6})	2.0	0.8^{++}	1.5	0.7^{++}	1.7

TABLE 9. Production and renewal rates of marrow B cell subsets in Bone Marrow Chimeras determined from *in vivo* BrdU labeling.

Bone Marrow was harvested from young or aged C57BL/6 mice and 3 X 10^6 T- and B-depleted marrow cells were injected i.v. into young or aged (C57BL X DBA2)F1 mice. Adoptive hosts were allowed to reconstitute >40 days. Chimeric bone marrow was harvested. The proportional representation of pro-, pre-, and immature B subsets was assessed by FACS, and multiplied by the marrow cell estimate of Osmond (149, 150) to obtain total numbers. Figures given for untreated mice are from young or aged C57BL6.

‡ BrdU labeling was performed in reciprocal young <-> aged chimeric mice. The proportion of BrdU labeled cells was determined by FACS and the numbers of BrdU labeled cells calculated by multiplying these proportions by the marrow cell estimate of Osmond (149). The regression coefficients of absolute and proportional labeling vs. time provide estimates of production and renewal rates respectively.

§ Means were compared (young *versus* aged or reciprocal chimeras *versus* control chimeras) using student's t-test. $^{+}$, $P<0.05$; $^{++}$, $P<0.01$

From Dr. Michael Cancro, University of Pennsylvania, personal communication.

4.6 Defects in the aged murine bone marrow microenvironment are sufficient to result in defects in B cell development.

Before completing this work, I considered it likely that both cell-intrinsic defects and defects in the bone marrow microenvironment contribute to the age-associated reductions in *rag* expression and numbers of pre-B cells. *In vitro* experiments have indicated defects in both developing precursors from aged mice and the bone marrow stromal microenvironment (93, 94, 101). However, *in vivo* analyses to determine if microenvironment or cell-intrinsic defects contributed to loss of *rag* expression and pre-B cells had not been conducted. An advantage of using *in vivo* adoptive transfers to compare microenvironment and cell-intrinsic defects is that the cells analyzed are generated from earlier precursors while residing in the bone marrow microenvironment. In addition, samples were analyzed within hours after harvest, rather than after days in culture. During culture, cells from young and aged mice may display defects that manifest themselves as cell-intrinsic defects but are actually established prior to harvest due to the *in vivo* microenvironment. This would occur if precursor cells denied growth and developmental factors *in vivo* required had not been cleared by apoptosis. During *in vitro* culture, these cells may not be able to recover from the lack of stimulus that was experienced in the animal, and may appear defective as they are already be destined for apoptosis.

In this work, I conducted adoptive transfers of cells from young donor mice into young and aged mice and found that the aged bone marrow microenvironment was sufficient to result in reduced *rag2* expression and numbers of pre-B cells (Chapter 2). The fact that bone marrow from young mice displayed reduced *rag2* expression and V(D)J recombinase activity in pro-B cells and reduced numbers of pre-B cells in the aged host indicates that the defect responsible for these changes is dictated by the age of the microenvironment and not by cell intrinsic properties. Interestingly, adoptive transfers of cells from young and aged mice into young recipient mice also indicated that the aged bone marrow microenvironment was the source of reduced *rag2* expression and numbers

of pre-B cells (Chapter 2). B cell precursors derived from bone marrow of young or aged mice had similar levels of *rag2* expression and numbers of pre-B cells in young hosts. In fact, the level of *rag2* expression in pro-B cells from aged mice (that was lower in aged donor mice than in young donor mice) was restored to young-like levels following adoptive transfer into young host (Chapter 2). Further support to indicate that microenvironment changes are responsible for the loss of pre-B cells is the finding that production rates of pre-B cells (as measured by BRDU labeling) are determined by the age of the adoptive host, rather than the marrow donor (Table 9) (Labrie et al. in preparation).

4.7 Loss of pre-B cells may be due to reduced number or function of stromal cells.

What is defective in the aged microenvironment that could result in reduced *rag2* expression and progression of pro-B cells in aged mice? A leading candidate is a reduction in the function or number of bone marrow stromal cells. B cell development is dependent upon both contact with and factors derived from bone marrow stromal cells. The stromal-derived factors IL-7, flt3L, SDF-1, and SCF are all either essential or strongly supportive for B cell development [as reviewed in (54)] and (46, 48, 53, 59). And while specific extrinsic factors that induce *rag* expression have yet to be identified, it remains possible that a reduction in the number or function of bone marrow stromal cells could reduce signals that regulate transcription factors and in turn, *rag* expression. It is also possible that the age-related decrease in *rag2* expression could be due to attenuation of as yet unknown inductive signals that work independent of transcription factors.

My observations of *rag*2-GFP expression (using NG transgenics) in aged mice are consistent with a reduction in numbers of stromal cells. The percent of pro-B cells that express *rag2* is reduced in aged mice, however, in pro-B cells that do express *rag2*, the average level of expression per cell is similar to that of young mice. This is displayed in Figure 8 where the data obtained from analysis of pro-B cells from young and aged mice show that cells that are GFP+ have similar levels of expression per cell regardless of age.

This could indicate that fewer pro-B cells receive signals and factors from the stromal microenvironment, but those that do receive the required factors develop normally. Therefore, pro-B cell development may be limited due to access to reduced numbers of stromal cells and the short-lived, secreted factors that they produce.

FIGURE 8. Per cell level of GFP expression in pro-B cells is similar in young and aged NG mice

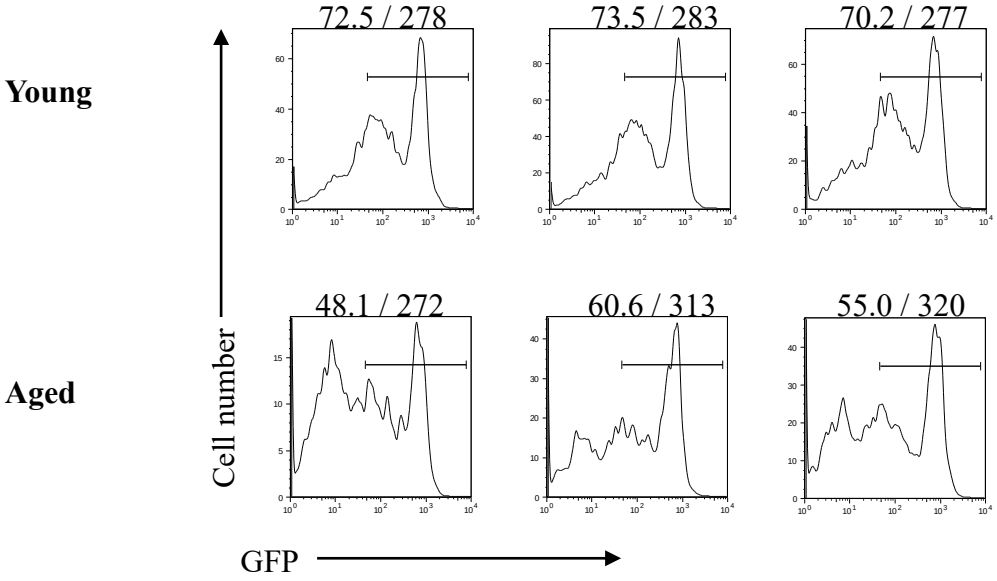


Figure 8. Per cell level of RAG2-GFP expression in pro-B cells is similar in young and aged NG mice. Displayed are histograms of GFP expression in pro-B cells harvested from representative young and aged NG mice. Numbers above graphs represent the percent of pro-B cells that are GFP+, and the mean fluorescent intensity of GFP per cell.

This hypothesis could be tested using cross-sectional immunohistochemical analyses of bone marrow from young and aged mice to determine if distribution of developing pro-B cells is reduced in bone marrow of aged mice. Another method would be immunohistochemical analysis of bone marrow from young and aged *rag2*-GFP mice to determine if *rag2* expression is restricted to fewer discrete areas in aged mice. If *rag2*-GFP mice were first crossed to *rag2*-/- mice to make *rag2*-/- *rag2*-GFP mice (that would therefore have no RAG2 protein), this would eliminate *rag2*-GFP expression in the bone marrow from B lineage cells past the pro-B cell stage. It is possible to make these mice because the *rag2*-GFP transgene is located on a different chromosome than the endogenous *rag* locus thus enabling us to make a mouse that has *rag2* knocked out at both loci and contains the RAG2-GFP transgene. [*rag1* and *rag2* are located on chromosome 2 and we have evidence that the *rag2*-GFP NG BAC is located on chromosome 8 (R. Gerstein data not shown)]. To avoid the time required to age these mice, bone marrow could be transferred from young *rag2*-/- *rag2*-GFP donors into young and aged recipients.

4.8 Polymorphisms influence the frequency of pre-B cells and re-circulated B cells in the bone marrow of aged mice.

One of goals of this research was to determine if the frequency of pre-B cells and other B cell subsets in the bone marrow of aged mice were influenced by genetic differences between inbred strains. A QTL analysis of 18 month old UM-HET3 mice identified two simple sequence length polymorphisms (SSLPs) that appear to influence frequencies of B cell subsets in the bone marrow (Chapter 3). My primary goal was to find a genomic marker corresponding to differences in frequencies of pre-B cells; this goal was accomplished. In addition, a marker associated with frequencies of re-circulated B cells was identified.

I found that the frequency of pre-B cells in the bone marrow is influenced by a specific SSLP, *D19Mit41* on chromosome 19. The polymorphism was determined to be between BALB/c and C57Bl/6 mice. The gene on chromosome 19 that influences the frequency of pre-B cells in aged bone marrow could be expressed in either B cell precursors or in stromal cells that provide essential support for B cell development. Stromal cells from aged mice are less supportive of B cell development than similar cells harvested from young mice (93, 94, 101) and we have found that the decline in pre-B cell frequency, *rag2* expression and V(D)J recombinase activity in aged mice are all conferred by the aged bone marrow microenvironment (Chapter 2). It is tempting to speculate that this genetic difference has an impact on developmental progression of pro-B cells. The genomic scan is not of sufficient resolution to identify specific polymorphic genes at this time: however, it is exciting to note that BLNK (also known as SLP-65) is located on chromosome 19. (BLNK is located within 15 Cm of the *D19Mit41* marker.) BLNK is an adapter protein that couples signaling from the pre-BCR-associated Syk kinase with downstream pathways essential to progression of pro-B cells to the pre-B stage (42). It is possible that a polymorphic difference in either the BLNK coding sequence or in regulatory elements could result in reduced formation of the pre-BCR signaling complex and impaired pre-B cell generation. Alternatively, the polymorphic gene could be in a different gene that is expressed in B cells or might be expressed in stromal cells that are required for B cell development.

The frequency of mature re-circulated B cells in the bone marrow in the aged mouse cohort is influenced by a specific SSLP, *D15Mit100* on chromosome 15 (Chapter 3). Re-circulating bone marrow B cells are mature B cells that resemble splenic follicular B cells and the B cells found in lymph nodes and peripheral blood. These cells are presumably an important part of immune surveillance and decline of this population with aging might underlie or reflect compromised mature B cell function. It is tempting to speculate that this polymorphism could occur in a gene involved in B cell homing, either expressed in B cells or in the bone marrow microenvironment.

In the case of both polymorphisms (*D15Mit100* and *D19Mit41),* the impact associated with the polymorphism could be age-dependent or age-independent. In this study a QTL analysis was conduced on 18 month-old mice. Bone marrow sampling is a terminal assay and as such samples were not obtained from the same cohort at a younger age. Thus we cannot formally exclude the possibility that the phenotypic differences observed here might occur at any age. Either scenario (age-dependent or age-independent) is of interest to us. Age-independent genomic differences between mice could lead to identification of genes that regulate B cell development that are currently unknown. Age-sensitive genomic differences have the potential to increase our understanding of mechanisms of immunosenesce in mice.

Further mapping studies could be used to more accurately map the polymorphic differences associated with each trait. Use of additional UM-HET3 mice would not be a practical approach; more refined mapping would require chromosomal cross-over events to determine precisely where on the chromosome the polymorphisms are located. A more practical approach is the use of recombinant inbred mice. The Jackson Laboratory offers 13 inbred strains of BALB/cBy X C57BL/6By recombinant inbred mice. Table 10 lists Strain Distribution Patterns for Chromosomes 15 and 19 in each of the 13 inbred strains recombinant strains. Use of these mice would enable further mapping to the linkages of the SSLP to a smaller region of the chromosome, thus enabling candidate genes to be identified.

TABLE 10. Recombinant Inbred Strain Distribution Patterns

Chromosome 15

CXB strain	1	2	3	4	5	6	7	8	9	10	11	12	13
D15Mit226	B	B	C	C	C	C	B	B	B	C	C	B	B
D15Mit121	B	B	C	B	B	C	B	C	C	B	C	B	C
D15Mit66	B	B	C	C	C	C	B	C	C	C	B	B	C
Tgn	B	B	C	B	B	C	B						
Ly6	B	B*	C	C*	B*	C*	B*	B	C	C	C	B	B
Ly6d	B	B	C	C	B	C	B						
Pmv17	B	B	C	C	B	C	B						
D15Mit71	C	B	C	C	B	C	B	C	C	C	B	B	B
Pdgf	B	C	B	C	C	B	C	B					
H30	B	B	C	C	B	C	B						
D15Mit159	B	C	C	C	B	C	B	B	C	C	B	B	B
D15Mit34	B	C	C	C	B	C	B	B	C	C	B	B	B
Gpd1	C*	B	C	C	B	B	C						
Smar Cd1	C	B	C	C	B	B	C						

Chromosome: 19

CXB strain	1	2	3	4	5	6	7	8	9	10	11	12	13
Mlviq2	B	B	B	B	B	B	B	C	B	C	B	B	B
D19Mit59	C	B	B	B	B	B	B	C	B	C	B	B	C
D19Mit109	C	B	B	B	B	B	B	C	B	C	B	B	C
D19Mit61	C	B	B	C	B	B	B	C	B	C	C	B	C
Ea4	C	B	B	C	B	B	B						
D19Mit23	C	B	B	C	B	B	B	C	B	C	C	B	C
D19Mit41	C	B	B	C	B		B	C	B	C	C	B	C
D19Mit85	C	B	B	C	B	B	B	C	B	C	C	B	C
D19Mit46	C	B	B	B	B	C	C	B	C	C	C	C	B
D19Mit40	C	B	B	B	B	B	B	B	B	B	C	B	B
D19Mit19	C	B	B	B	B	C	C	B	C	C	C	B	B
Cyp2 C29	C	B	B		B	C	C	B	C	C	C	B	B
Iapls3-8	C	B	C	C	C	C	C						
D19Mit123	C	B	B	C	B	C	B	B	B	C	B	B	B
D19Mit1	C	B	B	C	B	C	C	C	B	C	B	B	B
D19Mit34	C	B	B	C	B	C	C	C	B	C	B	B	
Xmv18	C	B	B	C	B	B	C						
D19Mit6	C	B	B	C	B	B	C	B	B	C	C	B	B

TABLE 10. Recombinant Inbred Strain Distribution Patterns. Displayed are the marker maps for chromosomes 15 and 19 for the 13 strains of recombinant inbred BALB/cBy x C57BL/6By strains available from Jackson Labs. *= inconsistent typing, and blank spaces are untyped. Table was replicated from http://www.informatics.jax.org.

4.9 Frequencies of peripheral T cell subsets are not correlated with loss of pre-B cells in aged mice.

Another important age-related change in the immune system is thymic involution, which begins shortly after adolescence. While B cell development is generally regarded as independent of T cells, pre-B cell numbers are lower in athymic mice than in age-matched controls and are partially restored following transfer of syngeneic T cells (97-99). In addition, numbers of pre-B cells are increased in aged and athymic mice following injection of either supernatant harvested from cultures of CD8 T cells activated *in vitro* or rIL-16 (97-99). These observations support the concept that reduced thymic output of T cells or altered peripheral T cell populations could contribute to lower pre-B cell numbers in aged mice. If this idea is correct, mice with more profound age-related alterations in T cell subsets or activation might be expected to suffer greater impairment of B cell development. We compared frequencies of peripheral T cells subsets known to be altered in aged mice (CD4M, CD8M, CD4P and CD8P in blood and spleen) and activation of splenic T cells following *in vitro* stimulation with anti-CD3 (Chapter 3). We found no correlation between the frequencies pre-B cells in the bone marrow and either the frequencies of T cell subsets or the extent of proliferation of splenic T cells. This lack of correlation suggests that the age-associated reduction in pre-B cells occur independent of alterations in T cell subsets in aged mice.

4.10 Frequency of re-circulated B cells is not correlated with loss of pre-B cells in aged mice.

In addition to a decline in the number of pre-B cells in aged mice, there is also an increase in the number of mature re-circulated B cells in the bone marrow (9, 91, 132). The requirements for homing, survival and persistence of mature B cells in the bone marrow are not well understood. It is possible that the increased number of re-circulated B cells in the bone marrow competes with developing precursors, therefore resulting in attenuated B cell development. If this were true, we would expect there to be an inverse correlation between frequencies of pre-B cells and re-circulated B cells in the bone

marrow. However, we did not observe an inverse correlation between re-circulated B cells and pre-B cell frequencies in the UM-HET3 aged cohort (Chapter 3). This could indicate that mature B cells do not inhibit earlier stages of B cell development or that this effect is not seen as early as 18 months of age. Analysis of pre-B cells and re-circulated B cells in bone marrow in inbred strains of mice indicate that the "aged phenotype" occurs more frequently in mice older than 18 months of age.

4.11 Frequencies of $B220^{LO}IgM^{+}$ cells are inversely correlated with frequencies of pre-B cells in aged mice.

We did not observe correlations between the frequency of pro- and pre-B cells with those of peripheral T cell subsets or re-circulated B cells in the UM-HET3 cohort. However, we did find that frequencies of both pro and pre-B cells were inversely correlated with the frequency of $B220^{int}IgM^{+}$ cells in the bone marrow (Chapter 3). Mice with higher numbers of $B220^{int}IgM^{+}$ cells tended to have fewer pro- and pre-B cells. Interestingly, the frequency of $B220^{int}IgM^{+}$ cells in the bone marrow was greater in mice that had more extensive age-associated changes in T cell subsets (higher frequencies of CD4M, CD8M in both blood and spleen and higher CD4P in spleen, and CD8P in blood).

These $B220^{int}IgM^{+}$ cells could be pre-memory plasma cells that have homed to the bone marrow. Further studies would be needed to evaluate whether these cells were elicited in response to the immunization at 15 months with turkey RBC. The mice were housed in SPF conditions and quarterly serology testing of sentinel mice found no evidence for viral infection during the course of these experiments, but we cannot completely exclude that increased $B220^{int}IgM^{+}$ cells could result from encounter with other infectious agents or environmental antigens of unknown kinds. These cells might also be activated autoimmune B cells. There is an increase in low affinity auto-reactive antibodies in aged mice (2, 3, 144, 145). It is possible that advanced T cell decline with age leads to a reduction in tolerance to self-antigens, resulting in an increase in activated

autoimmune B cells. The relationship of increased frequency of $B220^{int}IgM^{+}$ cells in the bone marrow and autoimmunity deserves further study.

4.12 Aging and B cell development in humans

Observations of aging defects in human B cell development are limited and conflicting.

Nunez et al. observed that the frequency of bone marrow surface $\square^{-}CD24^{+}$ cells (combined pro- and pre-B cells) decreases with age (fetus through 80 years old), and is greatly reduced at all ages compared to that of a fetus. However, this analysis included only 22 individuals, variability was high, and results of statistical analyses were not included (151). Nunez et al. also found expression of *rag1*, *rag2* and *TdT* in bone marrow pro-B cells ($CD34^{+}CD19^{+}$) from one fetus, four individuals 28 to 37 years of age, and one 62 year old individual (151). Expression of *rag1*, *rag2* and *TdT* were comparable in pro-B cells from fetal bone marrow and that of the 62 year old (151). This observation could indicate that *rag* expression is retained in aged humans; however, to my knowledge a quantitative comparison of *rag* expression in young (post-birth) and aged humans has not been conducted and the detection of *rag* expression in a single 62 year old individual may not be representative. McKenna et al. noted a significant decline in frequency of bone marrow B cell precursors (combined percent of pro-, pre-, and immature B cells) with age ($n= 432$, $r= -0.35$, $p< 0.001$). Variation was high between individuals of similar ages. Some adults, including those of advanced age, had a relatively high frequency of B cell precursors. Recently, Rossi et al. (152) determined that frequencies of B cell precursors do not change with age (ages 24 to 88 years). When frequencies of pro-, pre-, immature, naïve and mature B cells in the bone marrow were determined, differences between age groups were not statistically significant. However, variation between individuals was high, and number of individuals (total and within each age group) was not reported.

Additional work is required to determine if B cell development is diminished in aged humans. In the three studies mentioned above, the frequencies of B cell precursors are compared, rather than absolute cell numbers (which are very difficult to measure in human bone marrow). In addition, in these and most human studies, there are considerable genetic and environmental differences between individuals, and therefore changes in the number of B cell precursors as a result of age-related defects could be hard to detect. In each of the papers discussed above, the variability between individuals are high, and number of individuals are limited in one paper (151) or not stated in another (152). In these studies, it is possible that statistically significant differences between groups would not be observed unless the differences are of great magnitude. In the study with a large number of individuals, a significant decrease in combined pro-, pre- and immature B cells with age was observed (153). Therefore, it remains possible that age-related changes in human B cell development occur, and that they are more subtle than those of mice but still have significant impact on adaptive immunity. Alternatively, it may be true, as indicated in Rossi et al. that B cell development is not attenuated in aged humans (152).

Understanding age-associated changes in mice is still valuable as a model of aging, even if B cell progenitor frequencies are not reduced in humans. Stable frequencies of B cell precursors in aged humans do not rule out other age-associated defects. It is possible that Ig diversity is reduced in aged humans, if, in humans, *rag* expression decreases in pro-B cells (as I found in murine pro-B cells; Chapter 2). Despite the lack of observed defects in B cell development with age, B cell-mediated immunity is still greatly impaired in aged humans, as shown by decreased antibody response to vaccines and increased mortality to infection (2). Some of this impairment can be attributed to decreased T cell help (as discussed in the Introduction) but reduced diversity of B cell receptors could contribute as well. In addition, the three studies noted above found extensive variability in the frequency of B cell precursors between humans of similar advanced ages. It would be valuable to determine if lower frequencies of B cell

precursors are observed in individuals with reduced antibody response to vaccines and increased frequency of infections. This hypothesis could also be tested in aged mice. In addition, the use of mice could lead to the identification of genes that regulate age-associated defects in B cell development (such as those linked to the polymorphs found in our analyses [Chapter 3]). Polymorphic differences in the corresponding genes in humans may lead to therapeutics that enhance B cell development in aged humans or humans with other conditions that attenuate B cell development such as HIV, post-chemotherapy, post-bone marrow transplant, lymphomas and multiple myeloma (93, 153).

4.13 Summary and future goals

Here I have shown that expression of *rag2* and V(D)J recombinase activity are reduced in pro-B cells of aged mice and are correlated with the loss of pre-B cells. These defects are a result of the aged bone marrow microenvironment. In addition, pre-B cell frequencies in aged mice are influenced by a polymorphic difference between C57BL/6J and BALB/cJ mice. Furthermore, the percent of pre-B cells in aged mice is inversely related to the frequency of $B220^{int}IgM^{+}$ cells in the bone marrow and not correlated with frequencies of re-circulated B cells or peripheral T cell subsets.

While this work has provided several contributions to our understanding the effect of aging on B cell development, there are numerous issues that remain to be resolved. This includes determining if stromal cell numbers or functions are reduced in aged mice and further mapping of the polymorphisms located on chromosomes 15 and 19. Other future efforts might involve determining if the defect if *rag2* expression and V(D)J recombinase activity in aged mice is related in cause to another defect in gene expression in pro-B cells of aged mice. *λ5* and *E2A* gene products (E12 and E47) are reduced in pro-B cells of aged mice (91, 95, 100). It is possible that reduced expression of these B cell specific proteins are due to the same mechanism that results in loss of *rag2* expression and V(D)J recombinase activity. It would be valuable to next determine if reduced expression of *λ5*

and *E2A* are observed in all pro-B cells or if the reduction in expression is attributed to a reduced percent of pro-B cells that express these genes. Additionally it would be interesting to determine if *E2A*, *λ5*, and *rag2* expression occur in the same pro-B cells in aged mice, while other pro-B cells are devoid of expression of all three genes. Both of these questions could be tested by sorting GFP^+ and GFP^- pro-B cells from aged *rag2*-GFP mice (or aged wild type mice that have received adoptive transfers of bone marrow from *rag2*-GFP mice) and conducting RT-PCR or western blots for λ5, E12 and E47 expression or proteins.

Therefore, better understanding of the underlying mechanisms responsible for these defects in B cell development in mice should enable the design of experiments to address these changes in humans. Ultimately, this could lead to therapeutics that restore B cell production and improve the adaptive immune response to both pathogens and vaccines in aged humans.

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